Veterinär-Anatomisches Institut
der Vetsuisse-Fakultät Universität Zürich

Direktor *ad interim*: Prof. Dr. Mariusz P. Kowalewski, PhD

Arbeit unter wissenschaftlicher Betreuung von
Prof. Dr. Mariusz P. Kowalewski, PhD

Investigations towards Understanding the Importance of HIF1 Heterodimer in Regulating Steroidogenesis: A Methodological Approach

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

Bettina Lanfranchi
Tierärztin
von Poschiavo, Graubünden

genehmigt auf Antrag von

Prof. Dr. Mariusz P. Kowalewski, PhD, Referent
Prof. Dr. Max Gassmann, Korreferent

2020

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1 Zusammenfassung

Als Hauptproduzenten der Steroidhormone sind die Gonaden notwendig für die Erhaltung der Fortpflanzungsfähigkeit und funktionieren dabei unter einer reduzierten Sauerstoffsättigung. Das bedingt, dass die Aktivität des Hypoxie Induzierbareren Faktors 1 (HIF1) Heterodimers für die Steroidsynthese nötig ist. Als Teil des Komplexes, wirkt HIF1α dabei positiv auf die Steroidogenic Acute Regulatory (STAR) Protein-Expression ein, was *in vitro* in Granulosazellen unter einer reduzierten Sauerstoffkonzentration (10% O_2) bestätigt wurde. Die zugrundeliegenden molekularen Mechanismen bleiben unklar. Neuste Erkenntnisse haben nahegelegt, dass HIF1 an der Expression und Funktion von cJUN beteiligt ist, und möglicherweise die Aktivität des Co-Aktivators CBP/p300 beeinflusst. Dieser stabilisiert die Bindung von P-CREB an den *Star* Promoter. Um die mögliche Beteiligung von HIF1α auf die Funktionalität von CBP/p300 zu untersuchen, wurde in der vorgelegten Arbeit, u.a., eine Nachweismethode mittels eines CBP-Hämagglutinin (HA) Expression Vektors in KK1 Granulosazellen etabliert. Hierbei wurde ein neuer Regulationsweg aufgedeckt, in dem HIF1 die Expression und somit die Verfügbarkeit von CBP steuert. Dadurch wird die STAR-Expression reguliert. Eine Methode zur Immunpräzipitation des CBP-HA wurde ebenfalls etabliert. Weiterhin, die Untersuchungen mit mLTC1 Leydig Zellen haben gezeigt, dass den Granulosazellen ähnlich, HIF1α an der Regulation der STAR-Expression in diesen Zellen beteiligt ist.

2 Summary

As main producers of steroid hormones, gonads are essential for maintaining reproductive capacity. They function physiologically under a reduced oxygen tension, which entails the need of hypoxia inducible factor 1 (HIF1) heterodimers for steroid synthesis. Additionally, as a part of the complex, HIF1α acts as a positive regulator of steroidogenic acute regulatory (STAR) protein expression and function. This has been confirmed *in vitro* in granulosa cell cultures under reduced oxygen content (10% O_2). However, the underlying molecular mechanisms remain unclear. Recent findings suggest the involvement of HIF1 in positive regulation of cJUN expression and function, and possibly in regulating the activity of the co-activator protein, CBP/p300. The latter stabilises the binding of P-CREB to the *Star* promoter. Here, in order to elucidate the possible involvement of HIF1 heterodimers in regulating the activity of CBP, i.e., a detection system for CBP has been developed in KK1 granulosa cells, using the CBP-Hemagglutinin (HA) expression vector. Hereby, a new regulatory pathway has been unveiled involving the HIF1-dependent expression, and thereby the availability, of CBP. This, in turn, regulates the expression of STAR. Furthermore, an immunoprecipitation assay for CBP-HA protein has been developed. Additionally, investigations in mLTC1 Leydig cells revealed that similarly with granulosa cells, also in Leydig cells HIF1α is involved in regulating the expression of STAR.

3 Introduction

3.1 Steroid hormones – An overview

Steroid hormones are lipophilic, extracellular signalling molecules in multicellular organisms (Henley, Lindzey et al. 2005, Acconcia and Marino 2018). They are synthesized and secreted by specialized cells and exert specific biochemical actions on target cells. Multiple developmental and physiological processes are regulated by steroid hormones, determining they profound role in maintaining body homeostasis. The major sites of steroid hormone synthesis are in the steroidogenic cells of the adrenal gland and in gonads, i.e., in ovary and testis (Jerome F. Strauss III 2019). In addition, the central nervous system, the skin and, in several species, during pregnancy, the placenta presents an important steroidogenic source. According to their physiological function, steroid hormones are classified into following specific groups: mineralocorticoids, glucocorticoids, oestrogens, progestins, androgens, metabolites of vitamin D and neurosteroids (Acconcia and Marino 2018). Mineralocorticoids and glucocorticoids are collectively referred to as corticosteroids and are responsible for the regulation of the water-salt balance, stress response and carbohydrate metabolism. On the other hand, oestrogens, progestins and androgens are commonly known as sex steroids and are indispensable for maintaining reproductive capacity.

Steroid hormones act by modulating the gene expression (genomic pathway) within the target cell or by activating membrane-associated receptors and signalling cascades (non-genomic pathway) (Evans 1988, Aranda and Pascual 2001, Cato, Nestl et al. 2002). First, the genomic effects were identified and described. In this mechanism, steroid hormones diffuse through the cellular membrane due to their lipophilic character, and meet their specific receptors acting as nuclear receptors. Following their activation and nuclear translocation, transcriptomic effects can be exerted directly or indirectly. The direct effects are exerted through binding to specific DNA regions, i.e. hormone responsive elements (HRE), within promoter regions of genes. This is due to the activity of nuclear receptors as ligand-dependent transcription factors and/or cofactors (Evans 1988, Philips, Chalbos et al. 1993). Some of the nuclear receptors may also act as inhibitors of gene transcription (Higgins and Gehring 1978).

The non-genomic pathways are referred to as rapid signalling pathways, because they bypass the process of gene transcription. In this way, a steroid hormone action can be induced in minutes, whereas the genomic pathway typically takes hours (Norman, Mizwicki et al. 2004). Diverse signal transduction pathways have been reported to be affected by the non-genomic mediated actions of steroid hormones, such as the activity of mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), protein kinase B (AKT), nitric oxide (NO), protein kinase C (PKC), calcium (Ca^{2+}) flux and increase inositol triphosphate (IP3) levels. This way, steroid hormones can promote diverse cell processes such as apoptosis, survival and differentiation (Migliaccio, Castoria et al. 2000, Simoncini, Hafezi-Moghadam et al. 2000, Kousteni, Bellido et al. 2001). Importantly, it cannot be ruled out that the activation of signal transduction pathways by steroid hormones still affects gene expression (Wilkenfeld, Lin et al. 2018).

3.2 Biosynthesis of steroid hormones

The impact of the different classes of steroid hormones on the whole organism is diverse, nevertheless they use cholesterol as a common substrate (Werbin and Chaikoff 1961, Simpson and Mason 1976). Thus, cholesterol is converted in a cascade of enzymatic steps to the final active steroid hormone in a cell specific manner.

There are three different origins of cholesterol accessible for steroidogenic cells (Miller and Bose 2011):

1) *de novo* synthesis of cellular cholesterol in smooth endoplasmic reticulum (sER)
2) hydrolysis of cholesteryl esters stored in lipid droplets
3) uptake of lipoprotein-derived cholesteryl esters (high- or low-density; LDL, HDL) from the circulation via receptor mediated endocytosis

Although steroidogenic cells are able to synthesize cholesterol *de novo*, they preferentially utilize LDL and/or HDL from dietary sources to assure an adequate supply of cholesterol for steroid synthesis (Gwynne and Strauss 1982). A noteworthy control mechanism involved in HDL mediated uptake of cholesteryl esters was shown in rodents and involves the regulation of the availability of steroidogenic substrate by the cytoplasmic hormone sensitive lipase (HSL) (reviewed in (Kraemer and Shen 2002)). HSL is a multifunctional enzyme with cholesteryl ester hydrolase activity responsible for the release of cholesteryl esters internalized after HDL uptake or stored in lipid droplets. HSL is highly expressed in steroidogenic cells but also in several other tissues including the adipose tissue (Holm, Belfrage et al. 1987, Kraemer, Patel et al. 1993). In male mice HSL knockout results in oligospermia (Osuga, Ishibashi et al. 2000).

Regardless of the source of cholesterol, the first enzymatic step in steroidogenesis is identical in all steroidogenic cells and consists of the conversion of cholesterol to pregnenolone (P5). This enzymatic step is catalysed by the cytochrome p450 side chain cleavage enzyme (P450scc/CYP11A1), which is associated with the inner mitochondrial membrane (IMM) (Hall 1986). Therefore, a cell is defined as steroidogenic when P450ssc is expressed. However, cholesterol is a highly hydrophobic molecule (Haberland and Reynolds 1973) and in order to cross the aqueous intermembrane space between mitochondrial membranes it needs to be actively transported from the outer (OMM) to the inner mitochondrial membrane (IMM) (Davis and Garren 1968, Privalle, Crivello et al. 1983). This translocation occurs predominantly through the action of the steroidogenic acute regulatory (STAR) protein**.** Simple diffusion of cholesterol would not be rapid enough, therefore, the delivery of cholesterol by STAR is defined the rate-limiting step within the steroidogenic pathway (Lin, Sugawara et al. 1995).

Following the conversion of cholesterol to P5 it exits the mitochondria and is converted to progesterone (P4) in a step mediated by cytoplasmic, microsomal 3β-hydroxysteroid dehydrogenase (3βHSD/HSD3B) (Miller and Auchus 2011). The production of further steroids is determined by the activity of specific steroidogenic enzymes acting downstream of 3βHSD.

Interestingly, steroidogenic cells store very little steroids and depend, therefore, on their *de novo* synthesis. The regulation of steroidogenesis is primarily mediated by tropic hormones released by the pituitary, such as adrenocorticotropic hormone (ACTH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH). Tropic hormones regulate steroidogenesis by inducing either a chronic or acute steroidogenic response. The chronic response stimulates

growth of an endocrine gland resulting in increased steroidogenic capacity, e.g., by promoting the expression and function of steroidogenic enzymes, such as P450scc and 3βHSD. On the other hand, the acute response, which occurs within 15-60min following stimulation, acts predominantly via the cyclic adenosine monophosphate (cAMP)-protein kinase A (cAMP-PKA) pathway and involves increased expression and function of the STAR protein (Arakane, King et al. 1997, Manna, Dyson et al. 2009).

3.3 Gonadal physiology and steroidogenesis

The gonads represent primary reproductive organs and consist of ovaries in females and testes in males. Their major functions include: 1) the production of sex steroids, which are responsible for normal gonadal and reproductive function and development of secondary sex characteristics and, 2) the provision of mature gametes (oocytes or sperms). Gonadal steroidogenesis occurs, by definition, sexually dimorphic. This results in different hormonal effects and in diverging temporal patterns of regulation (Svechnikov and Soder 2008).

3.3.1 Ovary

Structurally the ovary consist of germline epithelium with various stages of follicle differentiation, a stroma composed predominantly of fibroblasts, and *hilus* serving for suspension of the ovary via the *mesovarium* and supplying vascular structures (Gougeon 2019).

The ovary is exposed to structural and functional changes within each reproductive cycle. This includes the maturation of oocytes enclosed in follicles passing through different phases of folliculogenesis until ovulation, and the formation of *corpus luteum*, a temporary endocrine gland acting as a source of P4 required for the establishment of pregnancy (Jerome F. Strauss III 2019). Ovarian follicles consist of an oocyte surrounded by specialized somatic cells, called granulosa cells, resting on a basal lamina. During folliculogenesis the granulosa cells go through morphological changes, divide intensely forming multiple layers, and specialize in two populations, either *cumulus oophorus* or mural granulosa cells (Khamsi and Roberge 2001). Whereas the mural cells rest on the basal lamina, cumulus cells surround the oocyte, and stay in a direct contact with it. There is a great bidirectional, cell-to-cell exchange of signalling molecules between granulosa and the oocyte, which is indispensable for regulation of follicular differentiation and growth, as well as for the oocyte to acquire the meiotic competence and for early embryogenesis (reviewed in (Eppig 2001)). Finally, the stroma cells surrounding the follicle form two, so-called, *theca layers: theca follicularis interna* and *externa* (Palma, Arganaraz et al. 2012).

Following ovulation, the *corpus luteum* is formed from the remaining wall-associated (mural) granulosa cells and the *theca interna* cells undergoing morpho-functional changes and giving rise to large and small lutein cells, respectively (Alila and Hansel 1984). These processes are accompanied by sequential changes of steroid hormone production regulated centrally by the hypothalamo-hypophyseal-gonadal axis. This system works as a closed loop and can be considered as entity. Within this loop, the function of hypophysis is controlled by the hypothalamic gonadotropic releasing hormone (GnRH). It regulates the secretion of pituitary hormones governing the gonadal function, like follicle-stimulating hormone (FSH), luteinizing hormone (LH), or prolactin (PRL). Finally, oestrogens govern the phase of follicular

development, whereas P4 dominates the luteal phase (Wood, Fata et al. 2007). In a case fertilisation would not occur, in most species, luteolysis, i.e., termination of the luteal phase, is brought upon by the auto-, para- and/or endocrine impact of the luteolytic prostaglandin 2α (PGF2α) (Pharriss and Wyngarden 1969).

Both, granulosa and *theca interna* cells, as well as following ovulation both populations of lutein cells, contribute to the ovarian steroidogenesis. The synthesis of oestrogens during the course of follicle development is based on a two-cell hypothesis (Hillier, Whitelaw et al. 1994). It depends on paracrine signalling and exchange of molecules between granulosa cell and *theca interna layer* (Hillier, Whitelaw et al. 1994). Within this functional interchange androgens are synthesized in theca cells and transferred to granulosa cells, where the conversion to oestrogen occurs. This is due to the exclusive expression of the enzyme steroid-17α-hydroxlase (CYP17) in *theca interna* cells, but not in granulosa cells. CYP17 mediates the conversion of P5 to dehydroepiandrosterone (DHEA) and androstenedione, both being precursors of oestrogens (Smyth, Miro et al. 1993). On the other hand, the expression of aromatase (CYP19A1) is restricted to granulosa cells (Whitelaw, Smyth et al. 1992). Androstenedione, produced initially in theca cells, is aromatized to oestrone and further converted to estradiol-17β (E2) under the control of FSH in granulosa cells (Jamnongjit and Hammes 2006). Following ovulation, the differentiation of granulosa- and theca cells into lutein cells is associated with a change in the expression of steroidogenic enzymes within granulosa and theca cells, and results in increased P450scc and 3βHSD expression in luteinized steroidogenic cells in order to produce large amounts of P4.

3.3.2 Testis

The testis is composed of seminiferous tubules, which are forming lobes, and an interstitium including Leydig cells, stroma cells and a vessel network (reviewed in (Ilacqua A. 2018)). In the seminiferous tubules, the germline epithelium is localized with different developmental stages of germ cells. The germ cells are referred to as spermatogonia, which during the process of spermatogenesis develop to spermatids, which further mature to spermatozoa in a process known as spermio(histo)genesis (reviewed in (Staub and Johnson 2018)). The maturing germ cells are accompanied by somatic Sertoli cells resting on a basement membrane supporting the tubules. Sertoli cells act as “nursery cells”, supporting the development of germ cells and building up the blood-testis barrier, which is required to protect the germ cells from the immune system and to create a microenvironment adequate for germ cells development (Chojnacka, Zarzycka et al. 2016). Within the interstitial compartment, Leydig cells are the major cells present, and form the endocrine portion of the testis (Tremblay 2015).

They are primarily controlled by the pulsatile release of LH, which acts via the LH receptor located in the cellular membrane, all under the control of GnRH (Lei, Mishra et al. 2001). Furthermore, several paracrine factors, including ghrelin and leptin, are important regulators of Leydig cell biology and function (reviewed in (Saez 1994)).

3.4 Steroidogenic Acute Regulatory (STAR) Protein

3.4.1 Acute regulator of steroidogenesis

Given the importance of STAR in the steroidogenic pathway by regulating the rate limiting step, STAR is the best candidate to fill the role as an acute regulator of steroidogenesis (Selvaraj, Stocco et al. 2018). First important observation towards understanding the acute role of STAR in regulating steroidogenesis, was following the blockage of protein translation by cycloheximide or puromocyin, which resulted in an inhibition of steroidogenesis in response to tropic hormones (Ferguson 1963, Davis and Garren 1968). This finding proved the need of *de novo* protein synthesis for the acute steroidogenic response. The ultimate characterization of STAR and close relationship to steroidogenesis was confirmed when the respective cDNA of STAR was purified and cloned in LH-stimulated MA-10 mouse Leydig cells in 1994 from the group of Douglas M. Stocco (Clark, Wells et al. 1994). Next, transient transfection of MA-10 with the cloned cDNA was sufficient enough to induce steroidogenesis in absence of tropic hormone stimulation (Clark, Wells et al. 1994). This was reconfirmed in COS-1 cells in which transfection with STAR cDNA in combination with the enzymes P450scc and adrenodoxin (a well characterized ferrodoxin, essential for electron transfer in the P450-mediated side chain cleavage (reviewed in (Hanukoglu and Jefcoate 1980)) resulted in similar outcome (Sugawara, Holt et al. 1995). These results demonstrated in a "cause and effect" relationship the role of STAR as an acute regulator of steroidogenesis (Lin, Sugawara et al. 1995, Sugawara, Holt et al. 1995).

The compelling evidence for the indispensable role of STAR in steroidogenesis, however, becomes more apparent when observing patients suffering from lipoid congenital adrenal hyperplasia (lipoid CAH), an autosomal recessive disorder with a mutation in the *Star* gene (Lin, Sugawara et al. 1995, Bose, Sugawara et al. 1996). Lipoid CAH is a potentially life-threatening condition, because both adrenal and gonadal steroid synthesis is severely impaired and the affected newborn die shortly after birth if no appropriate hormone substitution occurs. This is due to the lack of life sustaining hormones such as glucocorticoids and mineralocorticoids from the adrenal cortex (Lin, Sugawara et al. 1995, Bose, Sugawara et al. 1996). Targeted disruption of the *Star* gene in a mouse model showed similar pathophysiological characteristics to those seen in lipoid CAH of humans (Caron, Soo et al. 1997). Consequently, having seen STAR's key role in steroid synthesis a complete understanding of its tissue- and stimulus- specific expression (and function) became extremely important.

STAR is a 30kDa phosphoprotein synthesised from a 37kDa precursor, containing the N-terminal mitochondrial targeting sequence. As a result of mitochondrial import and processing a 32kDa intermediate product is formed, before it is processed to the mature 30kDa product localized within the matrix (Epstein and Orme-Johnson 1991, Stocco and Sodeman 1991, Artemenko, Zhao et al. 2001). The expression of STAR is confined to steroidogenic tissues and is present in the adrenal cortex, the gonads, the placenta of cow, pig and rodents, the brain and the skin (Pollack, Furth et al. 1997). Interestingly, although highly steroidogenic, human placenta lacks the expression of STAR, and no other cholesterol transporter has been identified in that tissue. The exact mechanism of cholesterol transport in the human placenta remains, thus, unknown (Strauss, Martinez et al. 1996).

As a key regulator of steroidogenesis, STAR is responsible for up to 85-90% of all cholesterol transport (Lin, Sugawara et al. 1995). The remaining 10-15% occur in STAR independent manner and relay on the process of passive diffusion or alternative, not yet defined, mechanisms (Clark, Combs et al. 1997). The mechanism by which STAR mediates cholesterol transfer has not yet been fully understood. Investigations of the biochemical structure of STAR at the OMM, suggest that STAR interacts with a protein complex, which promotes the cholesterol transfer (Bose, Whittal et al. 2008, Papadopoulos and Miller 2012, Rone, Midzak et al. 2012). It was reported that STAR interacts with voltage dependent anion channel 2 (VDAC2) and mitochondrial import receptor subunit (TOM22) at the mitochondria-associated endoplasmic reticulum membrane (MAM) (Prasad, Kaur et al. 2015, Rajapaksha, Kaur et al. 2016). This interaction is necessary for the mitochondrial processing of STAR into the mature protein and consequently for STAR's action in translocating cholesterol (Prasad, Kaur et al. 2015, Rajapaksha, Kaur et al. 2016). Importantly, every time STAR delivers cholesterol to the IMM, it is processed and inactivated. Therefore, to assure continuity of cholesterol transfer, *de novo* synthesis of the cytoplasmic 37kDa precursor is required. This further highlights the importance of the mechanisms controlling the transcription and translation of STAR protein.

3.4.2 Regulation of gonadal *Star* gene expression

The expression of the *Star* gene in the gonads is under the control of hypothalamic-pituitary-gonadal axis. FSH and LH are the main gonadotropins released by the pituitary gland affecting the gonads. They bind to their cognate 7-transmembrane G-protein-coupled receptors on the membrane of target cells within the gonads. The interaction with the receptor induces the activation of several signalling pathways, like those involving cAMP-dependent protein kinase A (PKA), phosphoinositid-phospholipase C-diacyglycerol-PKC-inositoltriphosphate (PLC-DAG-PKC-IP3), mitogen activated kinase (MAPK), arachidonic acid metabolites and calcium messenger system. Cross-talk between these pathways result in maximal increase of steroidogenic output. Importantly, activation of the cAMP-PKA pathway is the major route in tropic hormone dependent activation of STAR transcription defining the acute phase of steroid synthesis (Stocco, Wang et al. 2005, Manna, Dyson et al. 2009). Additionally, several studies have demonstrated a positive role of extracellular factors and/or signalling pathways on STAR expression, without affecting the levels of intracellular cAMP (Lin, Wang et al. 1998). These include growth factors, macrophage derived factors, arachidonic acid and its metabolites and chloride ions (Cl^-)(reviewed in (Stocco, Wang et al. 2005). Cumulatively, cross-talk between all signalling pathways provides fine tuning in the activation of STAR expression and function.

As the most important pathway regulating STAR expression and function, the cAMP-PKA pathway is mediated by an accumulation of cAMP in the cytoplasm and activation of PKA (Selstam, Rosberg et al. 1976). PKA consist of two regulatory and two catalytic subunits (Taylor, Buechler et al. 1990). The cAMP binds to its regulatory sites and induces detachment of the catalytic subunits, which then diffuse into the nucleus to phosphorylate specific transcription factors (Chin, Yang et al. 2002, Dyson, Kowalewski et al. 2009). Phosphorylation induces binding of a transcription factor to the corresponding DNA binding motif and promotes the recruitment of transcriptional co-activators. The assembly of transcription factors and co-factors activates the transcription machinery and initiates transcription. The cAMP-mediated

signalling leads to an increase of *Star* mRNA levels within 30min reaching maximal levels by 6h in the phase of the acute response (Clark, Combs et al. 1997).

There are two PKA subtypes involved in the regulation of STAR-mediated steroidogenesis, PKA type I and PKA type II. Both are activated upon cAMP accumulation within a steroidogenic cell. PKA type I is predominantly responsible for the transcription and translation of STAR, whereas type II PKA regulates the posttranscriptional modification, i.e. phosphorylation of STAR, in order to render the protein fully active (Dyson, Kowalewski et al. 2009). STAR harbours two conserved PKA phosphorylation sites located at Ser56/57 and Ser194/195. Mutation in these sites induces an impairment of STAR action leading to a reduced cholesterol transfer (Arakane, King et al. 1997). This highlights the importance of PKA-dependent phosphorylation in regulating the biological function of STAR, which is not given by the additional pathways involved in regulating STAR expression.

Besides the activation of STAR expression and function, the action of PKA results in the phosphorylation of proteins such as cholesteryl hydrolases. An important representative of cholesteryl hydrolases is HSL. As mentioned elsewhere (see 3.2) this enzyme is involved in regulating the availability of cholesterol for steroidogenesis, as well as in modulating the action of the cAMP-PKA pathway on the expression and function of STAR (Manna, Cohen-Tannoudji et al. 2013). A functional interplay between STAR and HSL was demonstrated to be involved in facilitating the trafficking of cholesterol and thus increasing steroidogenesis (Shen, Patel et al. 2003, Manna, Cohen-Tannoudji et al. 2013).

3.4.3 Characterization of the murine *Star* promoter and its activity

The complete *Star* promoter has a length of 3.6kb (Clark, Wells et al. 1994). Early studies used reporter gene assays to test the activity of the *Star* promoter and identified that the proximal 966bp 5'-flanking-region shows the capability of controlling both the basal and hormone-induced gene expression (Caron, et al. 1997a). Later a series of 5' deletion studies and site directed mutagenesis analyses were able to show that the region within the 250bp of the transcription start site (TSS) was sufficient for maximal promoter activity (Caron, et al. 1997a). Finally, alignment of *Star* promoter regions from various species revealed highly conserved sequence with specific regulatory DNA elements within the first 151bp upstream of the TSS (-151/-1) (Clark and Stocco 2014). This fragment possesses full-length promoter activity in response to cAMP (Manna, et al. 2002; Manna, et al. 2003b). It is noteworthy that although the activation of the *Star* gene is regulated by cAMP signalling pathway, the *Star* promoter lacks the palindromic consensus cAMP responsive element (CRE) (TGACGTCA) (Caron, Ikeda et al. 1997). However, three CRE-half sites **TGAC-** were found within the proximal promoter (Manna, Dyson et al. 2002) (Figure 1). Among those, the CRE2 (CRE/AP1) site was identified as the key element in cAMP-dependent regulation of STAR (Manna, Dyson et al. 2002, Manna, Wang et al. 2003, Manna, Eubank et al. 2004, Manna and Stocco 2007).

3.4.4 Transcription factors acting at the proximal *Star* promoter in response to cAMP - An overview

Several transcription factors were found to bind the highly conserved -151/-1 region of the *Star* promoter and influence *Star* gene expression. Among positive regulators are, e.g., cAMP responsive element-binding proteins (CREB) and CREB/CRE-modulator (CREM) family

members, steroidogenic factor-1 (SF-1), NUR77 (NR4A1), CCAAT/enhancer binding protein (C/EBPβ), and GATA-4 and -6, activator protein 1 (AP-1) family members (Manna, Wang et al. 2003). The strongest inhibitors of *Star* transcription are: Dosage-sensitive sex reversal-AHC critical region on the X chromosome (DAX-1) and ying yang 1 (YY1) (Figure 1) (Zazopoulos, Lalli et al. 1997, Nackley, Shea-Eaton et al. 2002). Besides, the CREB-binding protein (CBP) and its functional analogue p300 exhibit essential co-activator functions.

Among the transcriptional activators, CREB/CREM and AP-1 family members (JUN, FOS), were demonstrated as the most efficient in cAMP-dependent *Star* gene activation. They are responsible for the fine tuning by interacting with the CRE/AP-1 site (Manna and Stocco 2007). However, for the full cAMP responsiveness of the proximal *Star* promoter the binding of SF-1 and GATA-4 is crucial. The binding motifs of SF-1 and GATA-4 flank the CRE/AP-1 site and are therefore proposed to be involved in the stabilisation of the transcriptional complex (Wooton-Kee and Clark 2000, Manna, Wang et al. 2003). GATA-4 is phosphorylated by PKA to attain full activity and shows an important function in enhancing protein-protein interactions and the recruitment of CBP/p300 (Tremblay, Hamel et al. 2002, Hiroi, Christenson et al. 2004). The orphan nuclear receptor SF1 has been demonstrated to be highly expressed in steroidogenic cell types where it plays a pivotal role in the regulation of reproductive endocrine functions determining gonadal development and sexual differentiation (Luo, et al. 1994). Furthermore, the proximal promoter has a highly conserved CCAAT box that overlaps with CRE/AP-1 site, which is also recognized by C/EBPβ and plays central role in *Star* gene activation in granulosa cells of rodents (Yivgi-Ohana, Sher et al. 2009).

To sum up, the involvement of a variety of transcriptional activators highlights that besides the individual effects of these factors, their interaction, cooperation and relative abundance influence, in a species- and cell-type dependent manner, *Star* gene expression. Importantly, mutations at more than one place within the binding motifs frequently result in a stronger suppression of promoter activity when compared to just one site being affected (Manna, et al. 2003).

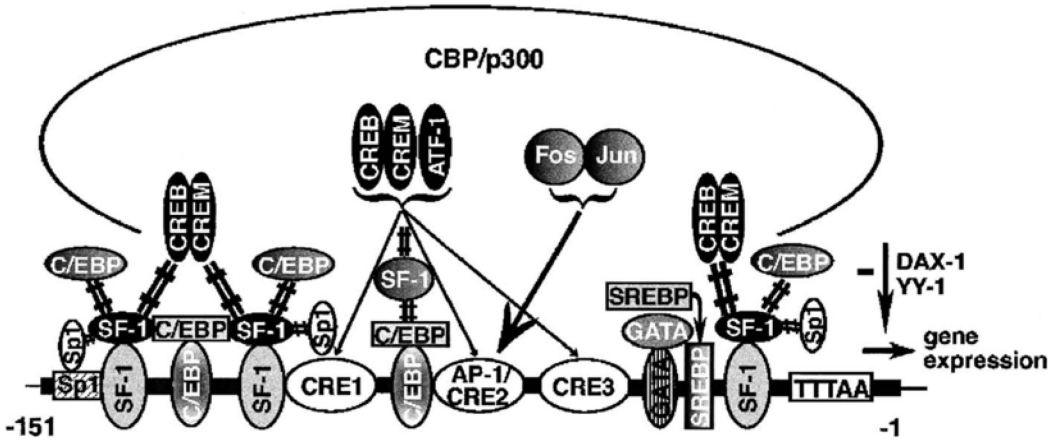


Figure 1: Schematic illustration of the murine proximal -151/-1bp *Star* promoter (Manna, Wang et al. 2003). Binding sites of the transcription factors involved in controlling *Star* gene transcription are demonstrated.

3.4.5 Selected important regulators of *Star* gene transcriptional activity

Cyclic AMP Response Element-Binding Protein (CREB) family

The members of the CREB family are the classical mediators of the cAMP signalling through the interaction with a conserved CRE (5'-TGCGTCA-3') site and consists of the protein members encoded by *Creb*, *Crem* and *Activating transcription factor 1* (*Atf-1*) genes (Montminy 1997). They belong to the basic leucine zipper (bZIP) family of transcription factors. bZIP proteins typically possess a DNA-binding domain and a leucine zipper coiled coil which enables homo- and heterodimerization among themselves or different family members (Vinson, Acharya et al. 2006).

CREB, is a 43kDa heavy protein, that has been shown to serve as principal agent in activating *Star* gene expression (Manna, Dyson et al. 2002, Clem, Hudson et al. 2005). Phosphorylation of CREB by PKA, PKC and other kinases at specific Ser133 residue renders the protein fully active and allows the recruitment of the essential co-activator CBP/p300 (Chrivia, Kwok et al. 1993, Mayr and Montminy 2001). CREB transcriptional activity occurs in a specific time-dependent manner and is clearly visible after 30min of stimulation with N6,2-dibutyryladenosine-3,5-cyclic monophosphate (dbcAMP) (Mayr and Montminy 2001, Clem, Hudson et al. 2005, Manna and Stocco 2007). After 60min the highest levels of phosphorylated CREB (P-CREB) can be detected, which slowly decrease, remaining however relatively high for up to 4h (Manna and Stocco 2007). The 30min needed for phosphorylation of CREB reflect the time for the catalysing subunits of PKA to reach maximal levels in the nucleus (Montminy 1997, Mayr and Montminy 2001).

As mentioned in 3.4.3, the proximal *Star* promoter does not possess a consensus CRE site (Manna, Dyson et al. 2002). Instead, three so-called CRE half-sites (CRE1, CRE2 and CRE3) have been identified and shown to interact with CREB/ATF family. Of these half-sites, CRE2 plays the major role in the initiation of *Star* transcription following hormonal stimulation (Manna, Dyson et al. 2002). Additionally, the CRE2 half-site overlaps with the binding motif of the AP-1 family members (CRE/AP-1 site) (Manna, Eubank et al. 2004). This suggests possible functional interaction between these two families of transcriptional factors coordinated by the CRE/AP-1 site (Manna and Stocco 2007).

Activator Protein 1 (AP-1) family

The AP-1 transcription factor family includes members of the FOS family (cFos, FosB, Fra-1 and Fra-2) and JUN family (cJun, JunB and JunD) (Angel and Karin 1991, Manna, Wang et al. 2003). Similarly, like the CREB/ATF-1 family, they belong to the bZIP proteins sharing structural and functional similarities. FOS members heterodimerize either with JUN family members or with members from the CREB/ATF-1 family, while the members of the JUN family can also function as homodimers (Hai and Curran 1991, Kerppola and Curran 1991). The dimerization composition affects their binding affinity and can alter in cell and stimulus specific manner the impact on *Star* gene activation (Manna and Stocco 2007). The AP-1 binding motif consists of the AP-1/TRE element with the DNA-Sequence 5'-TGA(C/G)TCA-3' and is overlapping with the CRE-2 site of the CREB/ATF-1 family (CRE/AP-1 site) (Figure 1). Phosphorylation in response to cAMP at Ser63 in cJUN and Thr325 in cFOS activates their full

binding capacity and facilitates the recruitment of co-activators, such as CBP/p300 (Clem, Hudson et al. 2005).

Functional comparison of JUN and FOS members show that among the AP-1 family, cJUN is the most potent trans-activator of *Star* gene (Manna, Eubank et al. 2004). Studies confirmed the essential role of cJUN by demonstrating its enhancing effect in PKC-mediated *Star* gene expression and steroidogenesis in mouse Leydig cells (Manna and Stocco 2008).

CJUN is frequently referred to as a quick responder in regulating transcriptional activity of *Star* gene (Manna and Stocco 2007). Thus, in contrast to CREB designated as a late responder, cJUN reacts rapidly upon stimulation with cAMP, reaching its maximal phosphorylation and, thus, transcriptional activity within 5-15 min, thereby acutely initiating the activity of *Star* promoter (Manna and Stocco 2007).

CREB-Binding Protein (CBP/p300)

The transcriptional co-activators, CBP and closely related p300 (CBP/p300), are believed to participate in the activities of hundreds of different transcription factors. CBP/p300 was first found as an interaction partner for CREB (Chrivia, Kwok et al. 1993) and later through its association with the adenoviral E1A oncoprotein (Eckner, Ewen et al. 1994, Arany, Newsome et al. 1995).

As a transcriptional co-activator, CBP exhibits important functions as an integrator promoting transcriptional activation (Figure 1). It is a large nuclear molecule of a molecular size of 265-300kDa. This allows it to have multiple functional domains and provides a scaffold which can interact with many transcription factors simultaneously, resulting in a stabilization of the transcriptional complex and contributing to transcriptional synergy (Janknecht 2002). CBP does not bind to the DNA itself but rather acts as a bridge, that links the components of the basal transcriptional machinery such as RNA polymerase II, TBP, TFIIB with the DNA-binding transcription factors (Vo and Goodman 2001). Additionally, CBP possesses histone acetyltransferase (HAT) activity that leads to chromatin remodelling making the DNA more accessible for transcription (Janknecht 2002). The phosphorylation of transcription factors by the activation of second messenger pathways allows the binding and recruitment of CBP/p300 (Parker, Ferreri et al. 1996). There are only limited amounts of intracellular CBP/p300 present, which results in a competition between different transcription factors for binding with CBP/p300. The latter presents an important regulatory mechanism in gene transcription (Arany, Huang et al. 1996, Manna and Stocco 2007, Liu, Wang et al. 2014).

The function of CBP can be repressed by the adenoviral E1A oncoprotein (Arany, Newsome et al. 1995). It interferes with the key C/H3 binding domain of CBP/p300 at which several key regulatory proteins interact, and inhibits its HAT activity (Arany, Newsome et al. 1995).

Studies have demonstrated the pivotal role of CBP in transcriptional regulation of *Star* gene (Manna and Stocco 2007). Upon phosphorylation of CREB, cJUN and cFOS by cAMP dependent mechanisms, an enhanced recruitment of CBP to the proximal *Star* promoter is observed (Manna and Stocco 2007). It has also been demonstrated that the overexpression of CBP/p300 potentiates the activity of CREB, FOS/JUN, C/EBPβ in *Star* gene activation. These

events can be reversed by the co-transfection with the adenoviral E1A oncoprotein (Manna and Stocco 2007).

Taken together CBP/p300 is one of the key regulators in the assembly and mobilization of the basal transcriptional machinery necessary for *Star* gene transcription, enabling specific genetic responses to diverse cellular signals within the steroidogenic cell.

3.5 Physiological aspects of reduced oxygenation (hypoxia)

3.5.1 Definition of a reduced oxygen content *in vivo* and *in vitro*

Oxygen (O_2) is the most abundant chemical element in the biosphere. It can easily cross the cellular membranes and initiate cellular responses directly or indirectly through intracellular O_2 sensors. It acts therefore as an important signalling molecule (Bartz and Piantadosi 2010).

For most organisms O_2 is fundamental to survive, as it plays key roles in life supporting processes such as the energy generation in form of ATP via oxidative phosphorylation in mitochondria (Mitchell and Moyle 1967). In addition, most enzymes depend on O_2 as a substrate. Therefore, it is of great importance for organisms to sustain the constant demands of O_2 by mechanisms of sensing and regulating O_2 levels in order to maintain body homeostasis.

Hypoxia is defined as a state where O_2 availability of an organ, tissue or a cell does not match levels necessary to maintain physiological O_2 tension and a biological reaction is caused. In other words, hypoxia occurs when the demand of O_2 surpasses its supply (Depping, Hagele et al. 2004, Wenger, Kurtcuoglu et al. 2015).

There is no exact pO_2 (see below) that determines the threshold of a hypoxic condition. The exact range rather needs to be determined for every specific characteristic of an organ, tissue or cell, and is therefore a relative term. The intensity of the biological reaction further depends on the duration of exceeded hypoxic condition.

For biological purposes, the O_2 content in the body is often given as O_2 partial pressure (pO_2, mmHg). That is pressure that O_2 exerts in a mixture of gases and corresponds to the total pressure it would have if it would occupy the whole volume on its own at constant temperature. It is the driving force for diffusion for O_2-molecules needed to traverse the body reaching its target location and therefore is directly linked to the O_2 availability. On the other hand, the O_2 concentration describes only the percentage of O_2 in the atmospheric volume. Thus, the O_2 concentration is 20.9% and corresponds at the sea level to a pO_2 of 159mmHg (in dry air). The O_2 concentration does not change with the altitude relating to the sea level, what changes at high altitudes is the pO_2. It falls exponentially together with the total atmospheric pressure corresponding to the physical law:

$$P = P_0 \cdot e^{-(0.127 \times a)}$$

P = atmospheric (total pressure) of air, a = increasing altitude in km, P_0 = atmospheric pressure at sea level (101.3kPa = 760mmHg)

Therefore, the hypoxic condition experienced in the mountains is defined as hypobaric hypoxia. It can be roughly calculated that 1% O_2 equals approximately 7.35mmHg pO_2.

The O_2 distribution *in vivo* follows a pO_2 gradient ranging from approximately 90mmHg in arterial blood and in lung alveoli to 40mmHg in mixed venous blood (Semenza 2010). However, the pO_2 can be even much lower at the end of a venous capillary reaching physiological levels of <10mmHg (Janknecht 2002, Wenger, Kurtcuoglu et al. 2015), e.g., in the renal papilla where a pO_2 of 1-2mmHg was measured (Lubbers and Baumgartl 1997). The tissue pO_2 depends strongly on the type of organ, its vascularization and its capacity to extract pO_2. For instance, values of 5-10mmHg in the myocardium of dogs (Moss 1968), 13mmHg in the cat retina (Linsenmeier and Braun 1992) and 10mmHg in rat thymus (Braun, Lanzen et al.

2001) were reported. Although these conditions appear to be hypoxic, these are the physiological pO_2 levels at which cell homeostasis is maintained.

In research, *in vitro* cell cultures are usually maintained in 20% O_2 (95% air and 5% CO_2), which under laboratory conditions is referred to as "normoxia". These are largely overestimated O_2 levels, which do not represent the endogenous pO_2 tension of an organ the cells come from. Therefore, *in vitro* conditions can be rather looked at as "hyperoxia". Knowing the drastic effects of an oxidative stress on cell physiology (Cross, Halliwell et al. 1987), it is important to determine the exact O_2 levels which correspond to the physiological conditions the cells origin from. This enables to study more accurately the proper functionality and physiology of the cells of interest. Therefore, in order to achieve this goal, the low O_2 contents *in vitro* are mimicked by lowering O_2 concentrations (normobaric hypoxia). Importantly, the cell density is further affecting their O_2 availability.

For research purposes, to distinguish between the various definitions of oxygenation levels *in vivo* and *in vitro*, the following summary and terminology can be helpful:

1) "normoxia" corresponding to atmospheric O_2 pressure (approximately 21%),
2) "tissue normoxia" or "physioxia" for the pO_2 estimated in different organs under physiological conditions,
3) "hypoxia" or "reduced O_2 content" representing a pO_2 lower than physioxia and indicative for status of lower oxygenation (Carreau, El Hafny-Rahbi et al. 2011).

3.5.2 Regulation of adaptive responses to hypoxic conditions

Taking into consideration the indispensable role of O_2 in maintenance of body homeostasis, exquisite mechanisms evolved to deal with conditions characterized by lower O_2 content with the intent to restore energy homeostasis. Once an organism or a cell is exposed to reduced O_2 tension, for example at high altitude, during fetal development or in pathophysiological conditions, where there is an impairment in vascular supply, it switches to mechanisms allowing adaptation and survival under these circumstances. These mechanisms can globally affect the physiology of the entire organism and occur within seconds. Global responses can include an increase in respiration or heart rate or changes of the blood distribution mediated by chemoreceptors, such as the carotid body, and the central and peripheral nervous system (Hopfl, Ogunshola et al. 2003). With a delay of minutes or hours, cellular components react to the lack of O_2 with changes in gene expression patterns. These alterations are regulated by transcriptional and post-transcriptional modifications and involve reactions induced by hypoxia inducible factors (HIFs) (Wang and Semenza 1995). Importantly, these interventions induced by HIFs not only have consequences on cellular level, but also exert effects on the systemic adaptation to hypoxia. A well-known example is the induction of the *erythropoetin (Epo)* – gene by HIF1 heterodimer (Semenza, Nejfelt et al. 1991). EPO was firstly described in liver cells exposed to hypoxic conditions. This leads to an increase of red blood cell production, which in turn expands the O_2 transporting capability of the blood circulation (Semenza, Nejfelt et al. 1991).

More recent research demonstrated existence of additional factors effecting gene expression under hypoxic conditions, e.g., through specific microRNA (miRNA), changes in chromatin

structure or modulations of the translational process (reviewed in (Rocha 2007)). The following chapter will focus on the role of HIFs.

3.5.3 Hypoxia Inducible Factors (HIF's)

HIFs are master regulators of adaptive responses to hypoxic conditions and are thought to be involved in the regulation of more than 100 genes (Fandrey and Gassmann 2009). HIFs are transcription factors active as heterodimers (HIF heterodimer) composed of approximately 120kDa alpha (α) and a 91-94kDa beta (β) subunit (Wang, Jiang et al. 1995). Both subunits belong to the basic helix-loop-helix (bHLH) transcription factors family containing a PER-ARNT-SIM (PAS) domain. The PAS domains (PAS-A and PAS-B) mediate the heterodimerization of the two subunits, whereas the amino terminal bHLH motif is required for the DNA binding to the corresponding DNA-sequences (hormone/hypoxia response element; HRE). The consensus HRE sequence is as follows: 5'-RCGTG-3` (R stands for A or G) (Jiang, Rue et al. 1996).

The β-subunit is identical to the aryl hydrocarbon nuclear translocator (ARNT) protein which was firstly identified as obligate dimerizing partner of the aryl hydrocarbon (e.g. dioxin) receptor (Forsythe, Jiang et al. 1996). It is stably expressed and localized within the nucleus. Its abundance and activity are not affected by O_2 content. The α subunit, on the other hand, contains the oxygen-dependent degradation domain (ODD) which confers O_2 regulated turnover (Huang, Arany et al. 1996, Huang, Gu et al. 1998). The ODD is a highly-conserved region that controls the activity and stability of the α subunits, as it contains the key proline (P) residues targeted for hydroxylation in normoxic conditions.

Three HIFα subunits were identified so far: HIF1α (Wang and Semenza 1995), HIF2α (also known and referred to as endothelial PAS domain -1 (EPAS1)) (Ema, Taya et al. 1997), and HIF3α (Gu, Moran et al. 1998). HIF1α and HIF2α share high sequence homology and function in similar, but non-redundant manner driving hypoxia induced target gene expression. Both carry an N- and C-terminal transactivation domain (N- and C-TAD) required for target gene selectivity (N-TAD) and co-activator recruitment (C-TAD) (Ema, Hirota et al. 1999, Hu, Sataur et al. 2007). Whether the response is HIF1α- and HIF2α-mediated, may depend on the duration of O_2 deprivation and is cell type specific. HIF1α appears to be responsible rather for the initial response to hypoxia (<24h), whereas HIF2α is supposed to begin to exert its effects after longer hypoxic periods (>24h, chronic hypoxia) (Koh and Powis 2012). Therefore, HIF2α induces rather the expression of genes involved in pluripotent stem cell maintenance and angiogenesis and has been described not to play roles in the activation of glycolytic genes (Hu, Wang et al. 2003). The function of HIF3α is less obvious and of divergent nature. Some studies indicate that the least splice variant, also referred to as inhibitory PAS domain protein (IPAS), act as dominant negative regulator by scavenging the β-subunit to prevent dimerization and DNA binding of the HIF1α/-2α (Makino, Kanopka et al. 2002, Maynard, Evans et al. 2005). Importantly, HIF1α is the best-studied and characterized subunit to date with the highest abundance. The indispensable role of HIF1α in adaptation to hypoxia is highlighted in knockout experiments demonstrating early embryonic lethality in mice (Iyer, Kotch et al. 1998).

The target genes of HIF1 heterodimer identified to date can be functionally categorized in: (1) genes that lead to an increase of O_2 delivery through a rise of erythropoiesis (inducing EPO

synthesis) and vasodilatation or angiogenesis (vascular endothelial growth factor (VEGF)-system), and (2) genes that decrease the O_2 consumption within the cell mainly involved in energy metabolism (e.g. glucose transporter GLUT1 or leptin, glycolytic enzymes) (Semenza 1998). Noteworthy, VEGF is one of the best-known targets of HIF1α (Liu, Cox et al. 1995, Forsythe, Jiang et al. 1996, Pagès and Pouysségur 2005). Importantly, HIF1 shows bimodal behaviour in affecting cell physiology. Thus, depending on the extent and duration of O_2 deprivation and on the cell type exposed, HIF1 heterodimer can either activate cell survival or cell death (Piret, Mottet et al. 2002, Wiesener and Maxwell 2003).

Regulation of HIF heterodimer activity

The biological activity of the HIF heterodimer is determined by the stability of the α subunit and is O_2 dependent (Salceda and Caro 1997). HIFα subunits are continuously transcribed and translated. However, they are unstable in the presence of O_2 and decay with a $T_{1/2}$ <5min due to posttranscriptional modifications reflecting cellular O_2 content (Wang, Jiang et al. 1995). Under normoxic conditions, prolyl hydroxylases domain (PHD) proteins hydroxylate two proline residues within the oxygen-dependent degradation domain (ODD) of the α subunit. Proline hydroxylation generates a binding site for the von-Hippel Lindau tumour suppressor (pVHL) E3 ubiquitinase complex, which poly-ubiquitinates the α subunit and thus targets it for proteasomal degradation (Huang, Gu et al. 1998). The most prominent PHD involved is PHD2, also known as egl nine homolog 1 (EGLN1). The PHDs require molecular O_2, α-ketoglutarate and ascorbate as substrates in order to conduct the modifications at proline residues. Their catalytic centre contains iron(II) (Fe(II)). The Fe(II) can be displaced or substituted by other transition metals such as cobalt (Co), nickel (Ni) and manganese (Mn) leading to loss of catalytic activity of the enzyme (Loenarz and Schofield 2011). Additionally, $CoCl_2$ is capable to bind to the pVHL binding domain within the ODD and prevents further steps of proteasome degradation (Yuan, Hilliard et al. 2003). Competitive antagonists of α-ketoglutarate, such as dimethyloxylylglycerin (DMOG) can also lead to an inactivation of the enzyme (Elvidge, Glenny et al. 2006). The inhibition of prolyl hydroxylases induces stabilization of HIFα subunit, leading to accumulation of HIFα within a cell.

Due to need of O_2 as a co-substrate, PHDs are cumulatively described as O_2-sensors and monitor constantly the intracellular O_2 content (Bruick 2003). A further mechanism involved in the regulation of the stability of α subunit involves the event of sumoylation by the **s**mall **u**biquitin related **mo**difier (SUMO) protein. Sumoylation is induced by hypoxia, promotes the stabilization of α subunit and enhances the transcriptional activity of HIF1 heterodimer (Bae, Jeong et al. 2004).

Finally, as the O_2 tension drops, HIFα is stabilized, accumulates and translocates to the nucleus, where it dimerizes with the HIFβ subunit to bind as HIF heterodimer to the HRE within the regulatory elements of target genes (Salceda and Caro 1997). Besides the stabilization of HIFα subunit, the transcriptional activation stays under control of changes in cellular O_2 contents. Thus, an enzyme known as factor inhibiting HIF (FIH) hydroxylates an asparagine residue within the C-TAD domain under normoxic conditions blocking, thereby, the interaction with CBP/p300 (Mahon, Hirota et al. 2001). The interaction of CBP/p300 with the C-TAD domain

is crucial for HIF-mediated gene induction (Arany, Huang et al. 1996). As mentioned elsewhere (see 3.4.5) CBP/p300 functions as important co-activator by interacting and stabilizing the transcription initiation complex and making the DNA more accessible through its HAT activity (reviewed in (Janknecht 2002)).

Importantly, multiple O_2 independent mechanisms affect the stabilization of HIFα facilitating thereby its activity. Molecules, like inflammatory mediators (Fukuda, Kelly et al. 2003), some cytokines (Gerber and Pober 2008) or growth factors (Herr, Keck et al. 2004) are involved in the enhancement of the expression of HIFα and DNA binding activity of HIF heterodimer, acting both under hypoxic, but also under normoxic conditions. One important signalling pathway involved in increasing the levels of HIF1α and acting in O_2 independent manner includes the phosphatidylinositol 3-kinase (PIK)/proteinkinase B (AKT) cascade, which activates rapamycin (mTOR) to increase HIF1α translation (Laughner, Taghavi et al. 2001, Fukuda, Hirota et al. 2002, Treins, Giorgetti-Peraldi et al. 2002).

Knowing that the activity of HIF heterodimer is tightly regulated on protein level, it is necessary to analyse the expression of α subunit on protein level in order to confirm the participation of HIF in a physiological or pathological process of interest (Salceda and Caro 1997).

3.6 Role of hypoxia in gonadal steroidogenesis

3.6.1 Ovary

Throughout every reproductive cycle, the ovary undergoes dynamic structural and functional changes, involving follicular growth and maturation, ovulation as well as *corpus luteum* formation, maintenance and regression. They are accompanied by continuous remodelling of complex vascular supply. These cyclic processes are regulated and modulated by pituitary and local hormones, like steroids, peptides and cytokines. In the underlying regulatory processes, the oxygenation of tissue, in particular of the follicular microenvironment, is critical to ensure normal ovarian function and finally reproductive success. Therefore, the following chapter will focus on elaborating the role of O_2 in regulating ovarian function.

3.6.1.1 Follicular vascular supply and oxygen distribution in the ovarian follicle

The follicular microenvironment is created by its specific vascular structure. During follicular development, the oocyte together with accompanying granulosa cells (i.e. *cumulus-oocyte complex* and mural granulosa cells) are separated by a follicular basement membrane from its surrounding stroma, which forms the *theca follicularis* layers, i.e., *theca interna* and *theca externa*. The vascular supply of a follicle is thereby restricted to the *theca follicularis interna* layer (Zeleznik, Schuler et al. 1981, Suzuki, Sasano et al. 1998) (Figure 2). The follicular basement membrane creates a functional borderline between vascularized and non-vascularized segments of a follicle. Accordingly, the follicular basement membrane was originally referred to as “blood-follicle barrier” (Zachariae 1958). This emphasizes that the growth and maturation of the oocyte and granulosa cell layers within the ovarian follicle occurs in an environment devoid of blood vessels.

The oocyte and the granulosa cells are both highly metabolic active and depend on the generation of energy in form of ATP via oxidative phosphorylation (Boland, Humpherson et al. 1994). The oocyte itself has only a small glycolytic capacity and uses preferentially pyruvate

as energy which implies that minimal level of O_2 consumption is needed for oocyte viability (Harris, Leese et al. 2009). Thus, the supply of nutrients, hormones and gases, including O_2, occurs via passive diffusion and gradient, as well as via cell-to-cell contact. The follicular basement membrane is selectively permeable and contributes to the bidirectional transport for providing means (Rodgers and Irving-Rodgers 2010). Transport by passive diffusion is only efficient for traversing short distances, further emphasizing that follicular microenvironment is characterized by low O_2 content and nutrient levels.

Several studies attempted to collect credible data reflecting accurately the dissolved O_2 concentrations within the antral follicle, e.g., by follicle fluid aspiration, and then rapidly assessing the dissolved O_2 content (Shalgi, Kraicer et al. 1972, Fraser, Baird et al. 1973, Fischer, Kunzel et al. 1992, Imoedemhe, Chan et al. 1993, Basini, Bianco et al. 2004, Redding, Bronlund et al. 2006). The measurements were, however, accompanied by analytic errors showing wide variations reaching from low to relatively high pO_2 and fraught with technical difficulties (Redding, Bronlund et al. 2006). Theoretical prediction using mathematical models have been developed to provide an alternate approach to estimate the pO_2 within the follicle (Gosden and Byatt-Smith 1986, Clark, Stokes et al. 2006, Redding, Bronlund et al. 2008, Clark and Stokes 2011). These models tried to illustrate accurately the O_2 diffusion rates regarding the follicular structures and suggested that the formation of an antrum is critical in improving the growth without creating a nutrient deficiency and an anoxic environment (Clark and Stokes 2011). Despite the antrum formation, mathematical calculations predicted relatively low pO_2 concentrations varying between 11-51mmHg (approximately 1.5-6.7% O_2) reaching a local minimum in the early pre-ovulatory phase (Redding, Bronlund et al. 2008).

Such low pO_2 can elicit a hypoxic response resulting in the induction of metabolic adaptions to maintain follicular homeostasis. An involvement of HIF-mediated gene expression is therefore of great probability. VEGF, as an important target of HIF1, plays a crucial role acting as proangiogenic factor in follicular development. It activates an enhanced vascularization in the follicle periphery and an increase of vessel permeability ensuring the supply of the growing follicle with hormones, O_2 and nutrients (Neeman, Abramovitch et al. 1997). Its essential role was further emphasized in studies in which the administration VEGF-receptor 2 (VEGFR-2) neutralizing antibodies resulted in reduced angiogenesis in the theca cell layer, antrum formation, granulosa cell proliferation and oestrogen production leading to an arrest of follicular development (Zimmermann, Hartman et al. 2003).

Following ovulation, the vascular supply within the ovary undergoes big changes. The follicular basement membrane breaks down during ovulation and blood vessels spread into follicular cavity fostering the process of luteinisation. During this time, the ruptured follicle is under hypoxia. This is due to formation of hematoma and still developing, insufficient vascularization (Amselgruber, Schafer et al. 1999, Manna, Dyson et al. 2002, Rizov, Andreeva et al. 2017). Finally, the developing *corpus luteum* is infiltrated by a complex vascular network, which enables the provision of virtually every lutein cell with access to a capillary (Redmer and Reynolds 1996). During this time, many angiogenic growth factors such as angiopoietins (ANGPTs), epidermal growth factor (EGF), fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), nerve growth factors (NGF), transforming growth factors (TGFs), tumour necrosis factors (TNFs) and VEGFs are involved in regulating luteal angiogenesis and

development (Reynolds, Grazul-Bilska et al. 1994, Redmer and Reynolds 1996, Goede, Schmidt et al. 1998). Among them, VEGF's are the most crucial luteotropic factors indispensable for the proper luteal formation (Ferrara, Chen et al. 1998, Fraser, Dickson et al. 2000, Fraser, Wilson et al. 2005, Zhang, Yin et al. 2011).

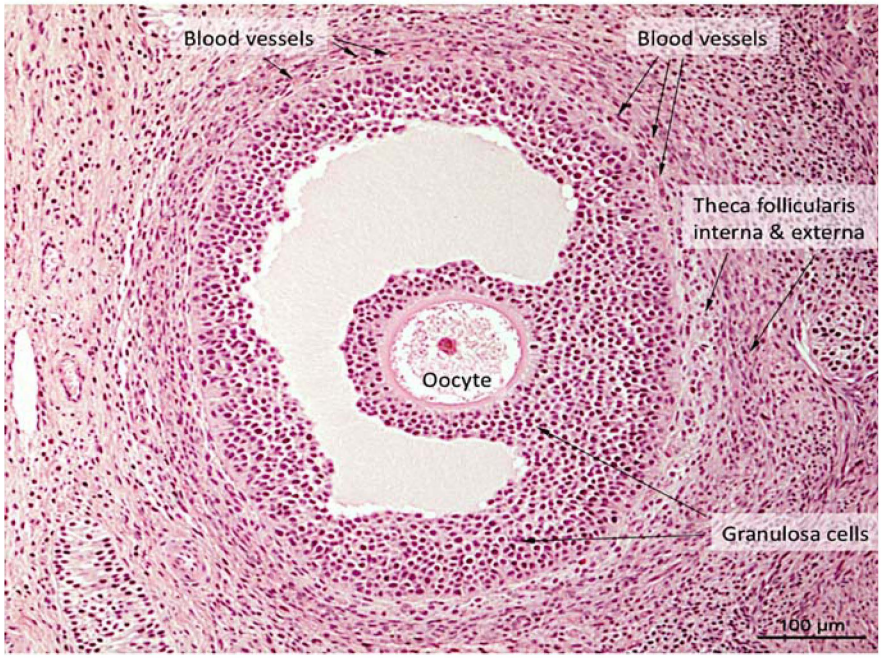


Figure 2: Representative microphotograph of a murine tertiary ovarian follicle. Indicated are the blood vessels, which are restricted to the *theca follicularis layers* with basement membrane constituting a borderline between vascularized and non-vascularized follicular compartments.

3.6.1.2 HIF1 heterodimer signalling in follicular development and ovulation

The facts described above (see 3.6.1.1), concerning the distribution of O_2 within ovarian follicles, emphasise that their development, ovulation and the early stages of luteal development, are physiologically accompanied by low O_2 conditions reflecting the physiological hypoxia. This suggests that HIF1 heterodimer-mediated gene expression is required to support the functionality of the ovarian follicle. Yet, studies have revealed that not only the low O_2 content is involved in the regulation of HIF1α expression during follicular development, but also pituitary hormones are involved in regulating HIF1α levels (Alam, Maizels et al. 2004, van den Driesche, Myers et al. 2008, Alam, Weck et al. 2009, Yalu, Oyesiji et al. 2015). Thus, e.g., treatment of primary rat granulosa cell culture with FSH in combination of a hydroxylase inhibitor $CoCl_2$, was shown to enhance HIF1 heterodimer transcriptional activity downstream of the PI3kinase/AKT/Rheb/mTor pathway (Alam, Maizels et al. 2004, Alam, Weck et al. 2009). The increased HIF1 activity was critical for the FSH-dependent upregulation of follicle differentiation markers such as luteinizing-hormone receptor (LHR), inhibin-α, microtubule-associated protein 2d (MAP2d) and the PKA type IIβ regulatory subunit in rat granulosa cell cultures (Alam, Maizels et al. 2004). This induction provides the key stimulus for the maturation of the ovarian follicle to the pre-ovulatory phenotype. The

molecular mechanisms behind the HIF1 heterodimer-mediated regulation of these genes is still not fully clarified. It appears, however, that HIF1 heterodimer is a component of a multiprotein complex necessary for the transcriptional regulation (Alam, Maizels et al. 2004). Besides FSH, also hCG (human chorionic gonadotropin) upregulates HIF1α expression in luteinized mouse and human granulosa cells *in vitro* and *in vivo* in presence of low O_2 content or $CoCl_2$ and seems to be involved in the HIF1 heterodimer mediated regulation of VEGF expression essential for follicular differentiation towards the *corpus luteum* formation (van den Driesche, Myers et al. 2008, Tam, Russell et al. 2010). Furthermore, the involvement of HIF1 heterodimer was implicated in studies demonstrating an increased HIF1α protein expression in granulosa cells of pre-ovulatory follicles of monkeys (Duncan, van den Driesche et al. 2008), porcine (Boonyaprakob, Gadsby et al. 2005) and mouse (Kim, Bagchi et al. 2009) reaching highest levels around the time of ovulation (Tam, Russell et al. 2010).

Finally, the indispensable role of HIF1 heterodimer signalling in follicular development and ovulation was shown by blocking HIF1α transcriptional activity with echinomycin, a selective inhibitor of HIF1 heterodimer DNA binding capacity, in pregnant mare serum gonadotropin (PMSG) - stimulated mice (Kim, Bagchi et al. 2009). Thus, treatment with echinomycin resulted in impaired ovulation and reduced gene expression of regulatory factors involved in the process of ovulation such as *Vegfa, Adamts-1* and *Edn-2* (Shindo, Kurihara et al. 2000, Hazzard, Xu et al. 2002, Palanisamy, Cheon et al. 2006). The anovulatory phenotype observed in HIF1-depleted mice was similar to that observed in animals lacking progesterone receptor (PGR) (Kim, Bagchi et al. 2009). PGR mediates the biological impact of P4 by activating a network of downstream target genes. Among these downstream targets, HIF1α, HIF2α and HIF1β were affected in the PGR knockout mice (Kim, Bagchi et al. 2009). This supports the fact that the stimulation of the PGR receptor is further involved in the regulation of HIF1α expression and HIF1 heterodimer activity and highlights the importance of HIF1 heterodimer signalling during final follicular development and ovulation.

Taken together, hypoxia and HIF1 heterodimer signalling are essential for the physiological process of final follicular development and ovulation, and support the formation of the *corpus luteum* by stimulating angiogenesis and cell proliferation (Nishimura and Okuda 2010, Jiang, Tsui et al. 2011).

3.6.1.3 HIF1 heterodimer signalling in ovarian steroidogenesis – Current state of knowledge

Since hypoxia, and in particular HIF1α, are essential for ovarian physiology, it appears plausible that they are involved in regulating ovarian steroidogenesis. Ovarian steroidogenesis includes the oestrogen production during follicular development by granulosa and *theca interna* cell layer, as well as P4 synthesis during the presence of the *corpus luteum* by theca- and granulosa lutein cells.

Indications of a possible role of hypoxia in steroidogenesis were provided by seeing a positive correlation of hypoxia- and hCG- induced up-regulation of proangiogentic factors like angiogenin (ANG) and VEGF, and increased P4 levels within the follicular fluid of pre-ovulatory follicles in women (Lee, Christenson et al. 1997, Koga, Osuga et al. 2000) (presented in 3.6.1.2). Early attempts to investigate the effects of hypoxia on steroidogenesis were carried

out in steroidogenic human placenta villi cell cultures (Gabbe and Villee 1971), as well as in rat, bovine and porcine luteinized granulosa and luteal cell cultures (Gafvels, Selstam et al. 1987, Koos and Feiertag 1989, Basini, Bianco et al. 2004, Nishimura, Sakumoto et al. 2006, Fadhillah, Yoshioka et al. 2014, Fadhillah, Yoshioka et al. 2017). Accordingly, both positive and negative effects were reported for the involvement of hypoxia in P4 synthesis. It appears that the impact of hypoxia on steroidogenic properties of cells is dependent on the O_2 content during cell culture, with extremely low O_2 levels (1% O_2) exerting severely diminishing effects. It needs, however, to be emphasized that the *in vitro* hypoxic conditions and the extent of hypoxia relating to the O_2 content in cell culture do not fully reflect the physiological hypoxia defined in pO_2 observed *in vivo*.

Thus, negative effects of hypoxia on P4 synthesis were initially demonstrated in investigations carried out in luteal cell cultures obtained from bovine corpora lutea of the mid-stage (Nishimura, Sakumoto et al. 2006, Jiang, Tsui et al. 2011). Different O_2 culture conditions, with decreasing O_2 concentration (20, 10, 5, 3, 1% O_2), reduced basal and LH-stimulated P4 production, reaching the lowest levels at 1% O_2. These inhibitory effects of severely reduced O_2 tension were correlated with suppression of the expression and enzymatic activity of P450scc (Nishimura, Sakumoto et al. 2006) and down regulation of STAR and 3βHSD under 1% O_2 (Jiang, Tsui et al. 2011). Furthermore, the diminishing effects of excessive exposure of cells to HIF1α on steroidogenic genes were shown in luteinized bovine granulosa cells cultured in the presence of $CoCl_2$ (Jiang, Tsui et al. 2011). These cells reacted similarly with a decreased expression and function of STAR and 3βHSD, resulting in a suppression of P4 synthesis (Jiang, Tsui et al. 2011). With this, a causality between hypoxia, overly expressed HIF1α and decreased steroidogenic output, was established. The inhibition of steroidogenesis was additionally linked to reduced activity (i.e. phosphorylation) of PKA (Jiang, Tsui et al. 2011). Similar effects were seen in whole rat corpus luteum cell cultures (Gafvels, Selstam et al. 1987). Notably, all these studies referred to the negative impact of either severely reduced O_2 conditions (3-1% O_2) and/or were observed in luteal cells from the mid/late-stage (Nishimura, Sakumoto et al. 2006, Jiang, Tsui et al. 2011). The decrease in P4 at the mid-luteal stage was explained as important factor for the regulation of the life span of the *corpus luteum* towards luteolytic cascade (Nishimura, Sakumoto et al. 2006). This was further emphasized by a hypoxia- and HIF1 heterodimer - mediated inhibition of P4 being associated with apoptotic activity of bovine lutein cells (Nishimura, Komiyama et al. 2008). The severe hypoxic conditions appear indeed to reflect the physiological conditions observed in the *corpus luteum* towards the end of luteal life span, i.e. during luteal regression, when the vascular supply is progressively restricted.

Conversely, it seems that luteinized granulosa cell cultures can benefit from a mild reduction of O_2 content, which more accurately reflects the microenvironment during follicular development, ovulation and the early stage of *corpus luteum* formation (Fischer, Kunzel et al. 1992). Thus, several results show that reduction of O_2 content under culture conditions to levels of 10% O_2 has positive effects on steroidogenesis in luteinized granulosa cells of several species, e.g., in swine, bovine, rat and mice (Koos and Feiertag 1989, Basini, Bianco et al. 2004, Fadhillah, Yoshioka et al. 2014, Kowalewski, Gram et al. 2015). These reduced oxygenated conditions (10% O_2) resulted in increased P4 output associated with elevated expression of

STAR (Fadhillah, Yoshioka et al. 2014, Kowalewski, Gram et al. 2015, Fadhillah, Yoshioka et al. 2017). Further reduction of the O_2 levels, however, markedly inhibited P4 accumulation in all these studies. The luteinisation, which is characterized by the change from oestrogen to P4 synthesis in cells undergoing the process, is accompanied by an acute upregulation of key steroidogenic proteins and enzymes, including STAR (Hillier 2001), the abundance and activity of which appear to profit from the reduced oxygenated conditions. Besides elevated P4 levels, the expression of HIF1α was upregulated in granulosa cells under 10% O_2, compared with cells cultured under 20% O_2 (Fadhillah, Yoshioka et al. 2014, Kowalewski, Gram et al. 2015).

The relationship between the reduced oxygenation (10% O_2), HIF1α, STAR and increased steroidogenesis was further elaborated in more detail in our research group using immortalized murine (KK1) granulosa cells treated with dbcAMP as a model (Kowalewski, Gram et al. 2015). Accordingly, we were able to show that concomitantly with exposure to reduced O_2 content in cell culture (10% O_2) the significantly increased steroidogenic response (P4 content) is associated with increased *Star* promoter activity, elevated *Star* mRNA levels and, finally, with increased STAR protein expression (Kowalewski, Gram et al. 2015). The expression of steroidogenic enzymes 3βHSD and P450scc was, however, not affected under the reduced oxygenated condition (10% O_2), applied in our study. Moreover, the reduced oxygenation-mediated effects were directly regulated by the transcriptional activity of HIF1 heterodimer (Kowalewski, Gram et al. 2015). This has been shown in experiments involving echinomycin as well as by knocking down the expression of HIF1α with small interfering (si)RNA, resulting in a significant suppression of basal- and dbcAMP-induced expression and function of STAR. Furthermore, a functional association of HIF1 heterodimer with the proximal (-151/-1) *Star* promoter was shown and a putative HIF1 heterodimer recognition site was identified (Kowalewski, Gram et al. 2015). In accordance with the above given data, more recently the direct role of HIF1 heterodimer in mediating STAR expression and function under reduced oxygenation (10% O_2) was shown in bovine luteinized granulosa cells (Fadhillah, Yoshioka et al. 2017). This study highlights that reduced oxygenation is important for steroidogenesis, which, in turn, is essential for luteinisation. However, further investigations are needed to understand the underlying regulatory mechanisms of HIF1 dependent STAR-mediated steroidogenesis.

Taken together, the above cited studies strongly support the idea that, when applied *in vitro,* a severely hypoxic milieu is not representative of the *in vivo* situation (Basini, Bianco et al. 2004), and rather partially reduced oxygenation appears more comparable with the physiological condition of the developing ovarian follicle.

Indeed, as shown in our experiments and results from other research groups, partially reduced oxygenated conditions support STAR-mediated steroidogenesis (Kowalewski, Gram et al. 2015, Fadhillah, Yoshioka et al. 2017). Noteworthy, partially reduced oxygenation was proved to positively affect tissues by inducing protection against apoptosis and increasing proliferation rates, whereas severely reduced O_2 contents frequently lead to apoptosis (Piret, Mottet et al. 2002, Greijer and van der Wall 2004).

3.7 Testis

3.7.1 Morphological and physiological characteristics contributing to the oxygen (O_2) distribution within the testis

The testicular function, which includes the process of spermatogenesis and the production of testosterone, is dependent on a strictly controlled environment considering temperature and vascular supply. The optimal environment for spermatogenesis is created by placing the testicles in most mammals in the scrotum where they are exposed to temperatures 2 to 7°C below body temperature, as well as by having a unique microcirculation operating fairly independent (Desjardins 1989). Both, the physiological location of the testis and the physiological morphology of the blood supply, affect its O_2 distribution.

Historically, testes were thought to operate on the "brink of hypoxia" (Setchell and Waites 1964). This was based on their anatomical and physiological characteristics. Firstly, the testicular blood flow rate is comparatively low in relation to the metabolic requirements of the organ and there is little to no possibility to increase the blood supply in moments of higher demands. Measurements of the physiological O_2 tension (pO_2) in the interstitium of the testis in rabbit (11.6mmHg), sheep (11.4mmHg), dog (8mmHg) and rat (15.2mmHg) indicate that testicular pO_2 levels are relatively low (Cross and Silver 1962, Free, Schluntz et al. 1976, Lysiak, Nguyen et al. 2000). Importantly, due to the high secretory activity and the high proliferation rate of germline cells, the testicular O_2 consumption is considered to be high. Whereas the Leydig cells are directly connected with the capillary bed, spermatogenic cells are dependent on O_2 diffusion from the basement of the seminiferous tubules. Therefore, the germline cells in the seminiferous tubules are apparently exposed to even lower O_2 tensions (Free, Schluntz et al. 1976). The pO_2 in the testicular interstitium is further determined by the O_2 saturation of the afferent arteries. The testicular artery and vein are in a very close proximity within the pampiniform plexus. The counter-current arrangement of these vessels suggests also a degree of gaseous exchange which, as a consequence, lowers the O_2 saturation in the arterial blood reaching the testis (Cross and Silver 1962). Additionally, it promotes an important thermoregulatory function in lowering the temperature of the testis. Importantly also the low temperature within the scrotum affects the O_2 saturation of the blood within the testis and must be taken into consideration. Algorithms for calculating blood gases based on known tissue temperature predict that a 1°C fall in temperature of blood decreases the pO_2 by 5mmHg (Bradley, Severinghaus et al. 1956). Hence, the lower temperature in the scrotum, at least in part, explains the low testicular interstitial and venous blood pO_2 (Free, Schluntz et al. 1976). This fact was further confirmed by comparing O_2 tensions in abdominal cryptorchidic and scrotal rat testes (Massie, Gomes et al. 1969). Thus, the reported O_2 tension in abdominal testis was twice as high as in scrotal testis and was suggested to be involved in testicular degeneration in the cryptorchidic testis (Massie, Gomes et al. 1969). Also local warming of the scrotum results in a rise of O_2 tension (Cross and Silver 1962).

In summary, the above cited data, including the observation of the physiological and anatomical characteristics and the direct measurements of interstitial pO_2 carried out across species emphasize that the testis has physiologically a low O_2 content. This also confirms the historical assumption that the testis works on the verge of hypoxia.

3.7.2 HIF heterodimer signalling in Leydig cells

Taken into consideration that the testis functions in an environment characterized by a low O_2 tension, an involvement of HIFs in maintaining tissue homeostasis needed for normal development of spermatozoa and steroidogenesis appears highly plausible.

First studies focussing on the examination of the presence and localization of HIFs in the testis confirmed a constitutive expression and stabilization of HIF1α, HIF2α and HIF3α were detectable only on the mRNA level (Powell, Elshtein et al. 2002, Palladino, Pirlamarla et al. 2011). These studies involved analyses of nuclear protein and mRNA extracts from whole testis of normoxic rats (i.e., exposed to normal air O_2 content). The protein levels of HIF1α were further increased in response to periods of testicular ischaemia (Powell, Elshtein et al. 2002). Immunohistochemistry of mice testis confirmed the expression of HIF1α and revealed strong signals localized in interstitial Leydig cells, whereas developing spermatozoa and surrounding Sertoli cells showed only a faint staining (Lysiak, Kirby et al. 2009). The expression of HIF1α was further demonstrated *in vitro* in Leydig cells (TM3), Sertoli cells (TM4), spermatogonia (GC-1spg) and spermatocytes (GC-2spd(ts)) cell cultures, which all responded with an induction of HIF1α protein in response to 1% O_2 culture conditions (Marti, Katschinski et al. 2002, Lysiak, Kirby et al. 2009). Further investigations of the activity and potential targets of HIF1 heterodimer in the testis were carried out in spermatozoa with the goal to clarify possible effects of HIF1 heterodimer signalling on the process of spermatogenesis (Marti, Katschinski et al. 2002). These cells were of particular interest, because they develop and maturate in the hypoxic milieu of seminiferous tubules. The O_2 reaches the lumen of the seminiferous tubules only by diffusion and the process of spermatogenesis is accompanied by high O_2 consumption, due to its high metabolic demand (Wenger and Katschinski 2005). The expression of a testis specific HIF1α isoform, HIF1α1.1, was reported, which was restricted to post meiotic spermatids of the mouse testis (Marti, Katschinski et al. 2002). Later, the same group of researchers identified another isoform of the HIF1α gene exclusively expressed in the human testis (hHIF1-αTe) (Depping, Hagele et al. 2004). The hHIF1-αTe isoform is considered to be a dominant negative regulator of normal HIF1 heterodimer activity. This isoform lacks the N-terminal 59 amino acids of the bHLH domain which makes it incapable of binding DNA, although an interaction with the β subunit can occur (Depping, Hagele et al. 2004). The expression of the dominant inhibitory HIF1α protein might be involved in silencing genes controlled by HIF1 heterodimer activity and stimulated under low pO_2 in the lumen of the seminiferous tubules. This regulatory mechanism is thought to promote downregulation of gene transcription in post meiotic cells (Steger 1999). The significance of this stringent regulation of HIF1 heterodimer function in spermatogenesis was further supported by findings in male mice with an inactive pVHL gene ($pVHL^{f/d}$ Cre mice). These mice showed a reduction in testicular weight, oligospermia and were infertile (Ma, Tessarollo et al. 2003). Furthermore, studies demonstrated that HIF2α is required for spermatogenesis as HIF2α knock out resulted in azoospermia (Scortegagna, Ding et al. 2003, Gruber, Mathew et al. 2010). The balance in HIF1α expression appears, thus, important for maintaining homeostasis of tissues.

With regard to the presence HIF1α and activity of HIF1 heterodimer in developing germ cells, HIF1 heterodimer and reduced O_2 tension were shown to exhibit also important regulatory mechanism in murine Leydig cells and positively affected steroidogenesis (Lysiak, Kirby et al.

2009). Accordingly, a stimulatory effect of reduced O_2 content (1% O_2) on the activity of 17α-hydroxylase-C17,20-lyase (P45017α) for conversion of P5 or P4 to dehydroepiandrosterone (DHEA) or androstenedione (A4) in mouse Leydig cells was demonstrated (Perkins, Hall et al. 1988). Conversely, however, oxidative stress perturbed the activity of the mitochondrial P450scc enzyme and inhibited the expression of STAR (Quinn and Payne 1984, Georgiou, Perkins et al. 1987, Diemer, Allen et al. 2003). Moreover, studies revealed a reduced oxygenation dependent VEGF-mediated increase in testosterone synthesis in murine TM3 Leydig cells *in vitro* (Hwang, Wang et al. 2007). The testosterone release was further enhanced by the application of intermittent reduced oxygenated conditions *in vivo* and *in vitro* in male rats (Hwang, Chen et al. 2009). Notably, the testosterone output stayed under the control of hCG, 8-Br-cAMP and forskolin in TM3 Leydig cells (Hwang, Chen et al. 2009). However, negative effects on testicular function and A4 production were observed after acute ischemic injuries such as testicular torsion (Lysiak, Turner et al. 2000, Turner, Bang et al. 2005).

Finally, the role of HIF1α in steroidogenesis was shown in TM3 Leydig cell cultures. When treated with $CoCl_2$ or transfected with HIF1α expressing vector, these cells reacted with an upregulation of the promoter activity of the mouse *HSD3β* gene (enzyme critical for the conversion of P5 to P4). The presence of a putative HRE element recognized by HIF1 heterodimer within the *HSD3β* promoter was identified (Lysiak, Kirby et al. 2009).

Cumulatively, it appears that the physiologically low O_2 tension in the testis is capable to stabilize HIF1α, which is necessary for the maintenance of normal testicular function including spermatogenesis and testosterone secretion. Most importantly, the significance of reduced O_2 tension and HIF1 heterodimer activity on STAR-mediated steroidogenesis remains to be elucidated.

3.8 Own contribution - Molecular mechanism of reduced oxygenation and HIF1 heterodimer-mediated regulation of STAR expression and steroidogenesis in KK1 granulosa cells

Taking into account our most recent findings on the impact of reduced O_2 content in cell cultures (10% O_2) and HIF1 heterodimer activity on STAR-mediated steroidogenesis in murine immortalized KK1 granulosa cells (Kowalewski, Gram et al. 2015) and luteinized bovine granulosa cells (Fadhillah, Yoshioka et al. 2017), my master thesis, accomplished in 2016, aimed to delineate molecular mechanisms involved in HIF1 heterodimer-mediated regulation of STAR expression and function in KK1 cells used as a steroidogenic model. The investigations of downstream mechanisms focussed on the effects of reduced oxygenation and HIF1 heterodimer signalling on the expression and activity, i.e. phosphorylation, of CREB and cJUN. Both transcription factors, CREB and cJUN, are among the most prominent regulators of cAMP/PKA-dependent STAR transcription and expression (compare chapter 3.4.5) In addition, the phosphorylation and activity of STAR under reduced oxygenated culture conditions (10% O_2), and the steroidogenic output (P4 synthesis), were examined (Lanfranchi 2016).

The KK1 cells origin from a granulosa cell tumour and were developed by using a transgenic mouse model expressing the SV40 T-antigen as the transgene and fragments of the inhibin-α-subunit as the appropriate tissue specific promoter (Kananen, Markkula et al. 1995). These cells

show fibroblast-like appearance and display a dose-dependent steroidogenic response upon stimulation with dbcAMP, hCG or forskolin (Kananen, Markkula et al. 1995, Havelock, Rainey et al. 2004). However, KK1 fail to show all characteristics of granulosa cells and present characteristics of luteinized cells as they respond only weakly to FSH and their P4 production is very high, due to partly loss of CYP19A1 expression (Kananen, Markkula et al. 1995).

In my experiments, KK1 cells were incubated under either 20% or 10% O_2 and supplemented with or without dbcAMP (0.1 mM) and echinomycin (5nM) for a time-period of 6h. The 6h time course was chosen because it is needed to induce the highest STAR expression and phosphorylation (P-STAR) as well as steroidogenic output (P4 synthesis) in response to dbcAMP stimulation. Cells were pre-incubated under respective experimental condition (20% or 10% O_2) overnight prior to stimulation experiments. All experiments were designed to be performed at least 3 times with cells from different passages.

The above described experimental conditions are in accordance with our previously published protocols (Kowalewski, Gram et al. 2015).

The following chapter will summarize the most important findings of the master thesis. The conclusions and hypotheses that emerged from the results became the basis of the goals that were elaborate in this doctoral thesis.

3.8.1 Characterization of cell culture model and validation of experimental conditions

Control experiments were carried out with the aim to characterize the maintenance of the steroidogenic responsiveness of the cell culture model and to validate the experimental conditions used in the main experiments. The intention of control experiments was to confirm the previously reported positive effects of reduced O_2 culture condition and HIF1 heterodimer activity on steroidogenic performance in granulosa cells (Kowalewski, Gram et al. 2015, Fadhillah, Yoshioka et al. 2017). In addition, the effects of 10% O_2 and HIF1 heterodimer on the activity, i.e. phosphorylation, of STAR (P-STAR) were investigated (Lanfranchi 2016). Thus, the steroidogenic capability, i.e. STAR expression and function, of KK1 granulosa cells was positively verified under 20% and 10% O_2. The PKA dependence was verified with H89 (specific inhibitor of PKA activity).

3.8.1.1 Effects of the reduced oxygen content (10% O_2) and HIF1 heterodimer activity on the expression and function of cJUN and CREB in KK1 cells

To gain a better understanding of the mechanism involved in HIF1 heterodimer-mediated STAR expression under reduced oxygen tension (10% O_2), the potential involvement of cJUN and CREB was determined (Lanfranchi 2016). The expression of cJUN and CREB was evaluated at mRNA and protein level. Their activity was assessed by measuring and comparing total and phosphorylated (i.e. cJUN/P-cJUN and CREB/P-CREB) protein amounts. Furthermore, the functionality of P-CREB was evaluated by using a transactivation assay (PathDetect CREB trans-reporting system).

For cJUN (Figure 3), under reduced oxygenation (10% O_2) a significant upregulation of the transcript and protein levels was observed. This was mirrored in an increased activation of cJUN represented in high levels of P-cJUN. These effects were abolished in response to the inhibition of HIF1 heterodimer activity by echinomycin.

An opposite effect, however, was observed for CREB (Figure 4) (Lanfranchi 2016). Whereas the amount of total CREB on mRNA and protein levels were not affected in response to dbcAMP and echinomycin, the treatment with echinomycin resulted in increased levels of P-CREB. Similar effects on CREB/P-CREB expression and activity were observed following the siRNA-mediated suppression on HIF1α expression. Transactivation assay further revealed that the high availability of P-CREB under echinomycin is associated with increased transcriptional activity of P-CREB. Despite its high transcriptional activation, P-CREB was not capable to induce *Star* transcription, which was reflected in a simultaneous decrease of STAR expression in response to HIF1 heterodimer activity blockage. This observation was perplexing and appeared to be *"against the dogma"* because CREB represents one of the most important regulators in basal and cAMP-mediated *Star* promoter activity (Manna, Dyson et al. 2002, Manna and Stocco 2007). Thus, its increased expression/activation is expected to be associated with increased transcriptional activity of the *Star* gene (Manna and Stocco 2007).

3.8.2 Conclusions and hypotheses

The results obtained from my master thesis provide first insights into possible molecular mechanism involved in HIF1-mediated STAR expression and steroidogenesis in immortalized murine KK1 granulosa cells. With regard to the previously shown positive role of HIF1 heterodimer under 10% O_2 in STAR-mediated steroidogenesis in granulosa cells (Kowalewski, Gram et al. 2015), this work (Lanfranchi 2016) unveiled possible mechanisms of the involvement of CREB and cJUN, as prominent transcriptional activators in underlying mechanisms.

Based on the observed results for cJUN and CREB the following hypotheses have been put forward:

1) HIF1 heterodimer is directly involved in regulating the expression and function of cJUN in KK1 granulosa cells.
2) HIF1 heterodimer is possibly involved in regulating the association of P-CREB with the proximal *Star* promoter by controlling the recruitment of CBP/p300.

Accordingly, the Figure 3 summarizes the proposed model of the involvement of cJUN in HIF1-mediated activation of STAR protein. The expression of cJUN was diminished by the presence of echinomycin, resulting in significantly lower levels of P-cJUN (transcriptionally active form). This results in a suppression of *Star* promoter activation, consequently decreasing STAR protein and steroidogenic output from granulosa cells. Notably, the increased phosphorylation of cJUN under moderately reduced oxygenation (10% O_2) can also be due to increased activation of PKA type I, the main phosphokinase involved in cAMP dependent *Star* gene activation (Dyson, Kowalewski et al. 2009). However, the impact of reduced oxygenation (10% O_2) and HIF1 heterodimer on the activity of PKA in KK1 granulosa cells, remains to be elucidated.

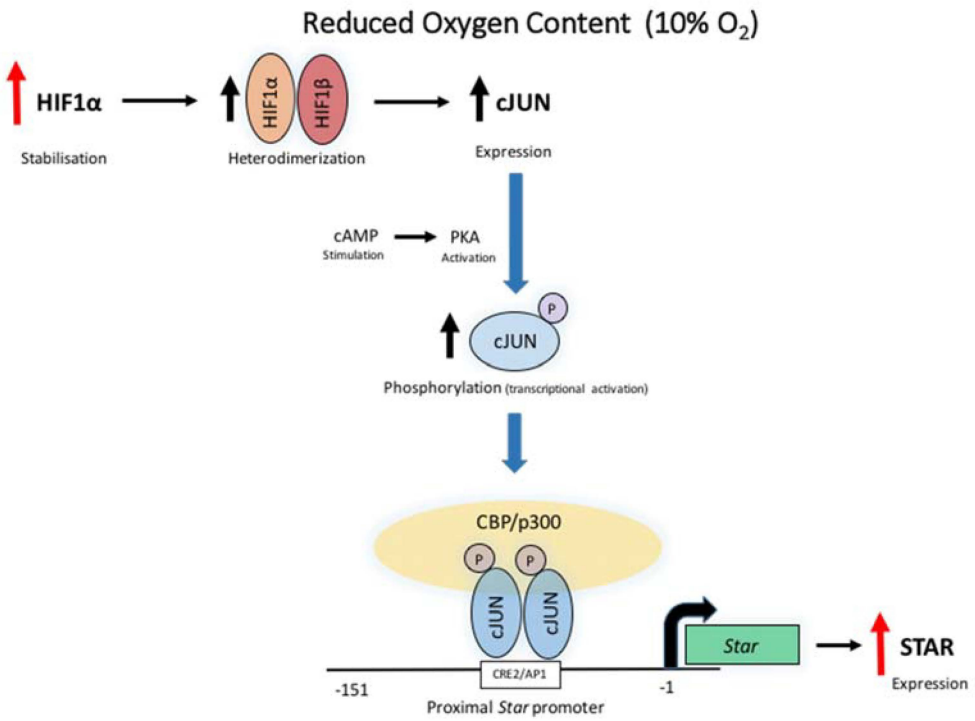


***Figure 3**:* Proposed model of the involvement of cJUN in HIF1-mediated activation of STAR protein expression and function under moderately reduced O_2 content in cell culture (10% O_2).

Conversely, the increased CREB and P-CREB levels under reduced oxygen content (10% O_2) imply a different regulatory mechanism. The most interesting finding from this part of my previous study (Lanfranchi 2016) is the highly elevated transcriptional activity of P-CREB observed in response to echinomycin treatment, associated with concomitantly decreased expression and function of STAR, resulting in suppression of P4 synthesis. This was unexpected, because the activity of CREB is absolutely required for the induction of STAR expression by cAMP/PKA-dependent mechanisms (Manna, Dyson et al. 2002, Manna and Stocco 2007). It seems that following the functional suppression of HIF1 heterodimer, P-CREB is not capable of binding to *Star* promoter and, instead, accumulates within the cell. Therefore, as illustrated in Figure 4 an involvement of the recruitment and activation of CBP/p300 by HIF1 heterodimer is suggested. In both HIF1 heterodimer- and P-CREB-mediated activation of transcription, CBP/p300 plays a fundamental role as co-activator (Arany, Huang et al. 1996, Manna and Stocco 2007). A direct interplay between P-CREB, HIF1 heterodimer and CBP/p300 was shown, e.g., in the *Ldha (lactate dehydrogenase A*) promoter (Firth, Ebert et al. 1995), and was also suggested for the *Vegf* promoter (Wu, Zhau et al. 2007).

Cumulatively, it is suggested that HIF1 heterodimer regulates the recruitment of CBP/p300 in order to stabilize the interaction of P-CREB with the proximal *Star* promoter needed to induce transcriptional activation under reduced oxygen content (10% O_2). This hypothesis still needs to be verified.

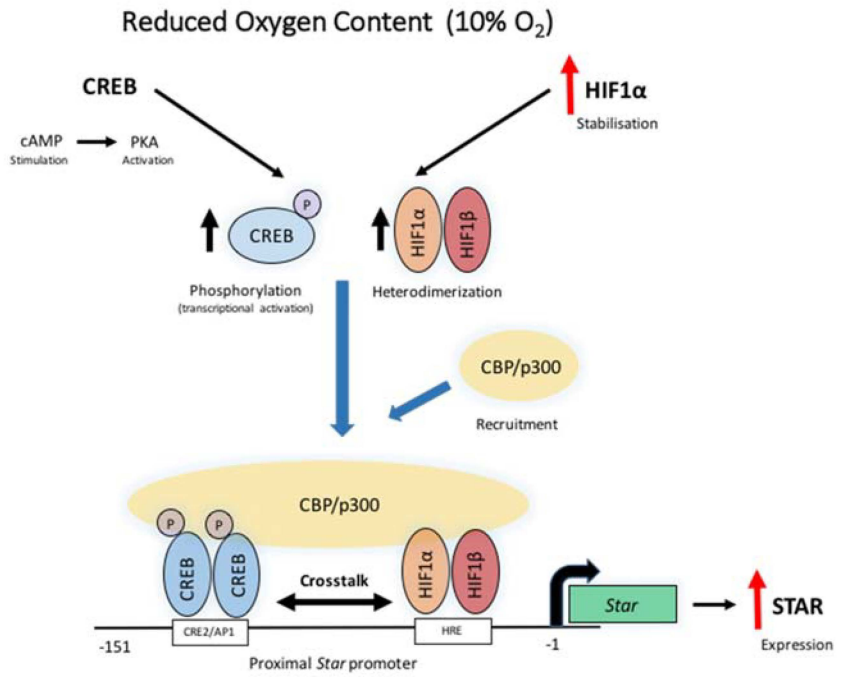


***Figure 4**:* Proposed hypothetical model illustrating the possible interaction between HIF1α, CREB and CBP/p300 in activation of STAR expression and function reduced O_2 content (10% O_2) in cell culture.

4 Aims of the doctoral thesis

Based on the above presented hypothesis derived from my master thesis that CBP is actively involved in HIF1-dependent STAR expression in steroidogenic cells under moderately reduced O_2 content in cell culture (10% O_2), the present thesis aims to establish a detection system for CBP protein expression and function in immortalized KK1 granulosa cells. The anticipated results will serve as a basis for future functional studies regarding the involvement of CBP in HIF1-dependent activation of STAR expression and function in steroidogenic cells.

In particular, the thesis will focus on the following aspects:

1) Assessment of CBP expression in steroidogenic KK1 granulosa cells following suppression of transcriptional activity of HIF1 heterodimer under normoxic (20% O_2) and reduced O_2 conditions (10% O_2)

2) Establishment of methods related to:
    a. Overexpression of CBP in KK1 granulosa cells for its immunodetection
    b. Immunoprecipitation of CBP

5 Material and methods

5.1 Cell lines and culture conditions

Table 1: Cell lines involved in the project

Name	Origin
KK1	Immortalized murine ovarian granulosa cell line derived from the ovarian tumour of a transgenic mice expressing the SV40 T-antigen under control of the mouse inhibin-α promoter (Kananen, Markkula et al. 1995); kindly provided by Dr. Ilpo Huhtaniemi, Hammersmith Campus, Imperial College, London, UK.
mLTC1	Mouse testicular Leydig cell tumour cell line derived from Mus musculus strain C57BL/6, designated M548OP (Rebois 1982); kindly provided by Dr. Jacques J. Tremblay, Department of Obsterics, Gynecology and Reproduction, Faculty of Medicine, Université Laval, Quebec, CA.
HEK293T	Human embryonic kidney cells (HEK) transformed with large T-antigen.

KK1 and mLTC1 cells were cultured in a humidified Incubator (Forma Steri-Cycle CO_2 Incubator, Model 371, Thermo Fisher Scientific AG, Reinach, CH) at 37°C and 5% CO_2. The culture medium was changed as required. As soon as 90-95% confluence was reached, cells were passaged and seeded into 6-well plates (TPP®, Trasadingen, CH) or into petri dishes (Sterilin Limited, Newport, UK) depending on the experimental condition. For splitting, cells were washed with sterile 1x phosphate buffered saline (PBS; Eurobio, Les Ulis, FR) without Mg and Ca and incubated with 1x trypsin (Gibco, Thermo Fisher Scientific AG, CH) for max. 2min at ambient temperature to allow the cells to detach. For stimulation experiments, approx. 1Mio cells were seeded into 6-well plates 24h prior to experiment.

Table 2: Cell culture media

Cell line	Composition
KK1	DMEM-Ham's F12 (Bioconcept Ltd., Allschwil, CH), 10% heat inactivated fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific AG) 15.4 mM sodiumhydrogen carbonate ($NaHCO_3$, Sigma Aldrich Chemie GmbH, Buchs, CH) 1% gentamycin (PAN Biotech GmbH, Aidenbach, DE) 1% L-glutamine (Gibco, Thermo Fisher Scientific AG)
mLTC1	RPMI-1640 (Bioconcept) 10% heat inactivated FBS 1% penicillin/streptomycin (100U/ml) (Sigma Aldrich Chemie GmbH) 1% L-glutamine
HEK293T	DMEM-Ham's F12 10% heat inactivated FBS 1% penicillin/streptomycin (all from Gibco, Thermo Fisher Scientific AG)

5.1.1 Stimulation protocol – Experimental set up

For stimulation experiments cells were seeded into 6-well plates (TPP®) 24h prior to experiments in order to achieve 80-90% confluence on the next day. During this time cells were pre-incubated under respective experimental conditions (20% or 10% O_2). The incubation under 10% O_2 was performed in incubator C16 (REF 1152, Labotec, Labor Technik Göttingen, Göttingen, DE). After 24h the serum containing culture medium was removed, cells were washed once with sterile 1x PBS and serum-free medium was added (stimulation medium), preconditioned 24h before stimulation experiments under respective culture conditions. For experiments determining STAR expression, stimulation experiments were performed over a time period of 6h. This duration was applied based on previous experiments showing highest output of STAR, P-STAR and P4 in steroidogenic cells upon stimulation with N6,2-dibutyryladenosine-3,5-cyclic monophosphate (dbcAMP) (Manna and Stocco 2007, Kowalewski, Dyson et al. 2010, Kowalewski, Gram et al. 2015).

5.2 Western Blot analysis

All buffers and solutions mentioned in the following chapter are described in detail in section 10.1 “Buffers and Solutions”.

The protein isolation was carried out in accordance with previously published reports (Gram, Buchler et al. 2013, Kowalewski, Gram et al. 2015). Thus, following stimulation, cells were rinsed with ice-cold 1x PBS and harvested using NET-2 lysis buffer (50mM Tris-HCl, 300mM NaCl, 0.05% NP-40, pH 7.4) containing 10µl/ml Protease Inhibitor cocktail (Sigma Aldrich Chemie GmbH). The whole cell lysate was transferred into a 1.5ml Eppendorf reaction tube. For homogenisation, the lysate was treated with a sonic distributor (Vibra-Cell, Newton, CT, USA), 75 Watt (40% efficiency) for 15sec, on ice. Afterwards, protein concentrations were measured in semimicrovolume cuvettes (BioRad Europe GmbH, Basel, CH) by Bradford assay (Bradford Quick Start, BioRad) with a UV-spectrophotometer (SmartSpec™ Plus Spectrophotometer, BioRad). The protein homogenates were further diluted in NET-2 lysis buffer and 4x Sample Buffer in order to reach the desired final concentration. Prior to loading the protein samples on polyacrylamide gel, they were heated for 10min at 95°C.

Separation of proteins by Sodium-Dodecyl-Sulfate-Polyacrylamid-Gel-Electrophoresis (SDS-PAGE) was completed using 6-12% polyacrylamide gels. The percentage of polyacrylamide gel was selected depending on the molecular size of the protein of interest. The stacking gel used had a percentage of 5%. In each gel slot 20-30µg of total protein were loaded; a molecular weight marker (PrecisionPlusProtein™, Dual colour Standards, BioRad) ranging from 10kDa to 250kDa was applied. Separation of proteins was carried out in an electrophoresis chamber (Mini Protean® and Tetracell, BioRad) filled with Running Buffer (details see 10.1). Firstly, a current of 80V was applied, allowing the collection of the proteins in the stacking gel. Afterwards, the current was increased to 120V for separation of proteins in the separation gel. Subsequently, the proteins were transferred (blotted) onto a methanol-activated polyvinylidene fluoride (PVDF) membrane (BioRad). The protein transfer was performed in a wet blotting chamber (Criterion Blotter, BioRad) filled with ice cold transfer buffer (details in chapter 10.1) for 1h at 100V. After blotting, membranes were incubated for 1h in 5% low-fat milk powder-PBS/ 0.25% Tween 20 (PBST) buffer-solution in order to avoid non-specific antibody binding

to membrane. Then the milk-PBST-solution was replaced by the respective primary antibody diluted in 2.5% low-fat milk powder diluted in PBST. Membranes were incubated with the primary antibody overnight at 4°C. This was followed with 3x 10min wash in ice cold PBST at ambient temperature. Thereafter, the membranes were incubated with a specific horseradish peroxidase (HRP)-conjugated secondary antibody diluted in 2.5% low-fat/PBST for 1h at ambient temperature. After incubation with the secondary antibody, another washing steps followed, 5x 10min with PBST at ambient temperature to remove the unbound secondary antibody. The chemiluminescent signals were captured with molecular imager ChemiDOC XRS+ system and IMAGE Lab Software (BioRad) using a chemiluminescent substrate (SuperSignal™ West Pico or SuperSignal West Femnto Chemiluminescent Substrate, Thermo Fisher Scientific AG) for 5min according to manufactures instructions to induce signals. Every membrane was re-probed with an anti-B-ACTIN antibody for loading control. Therefore, the membranes were stripped with 0.1M Glycine (pH 2.6; Biosolve Chimie SARL, Dieuze, FR) for 1h at ambient temperature, before the same detection steps of B-ACTIN as described above followed.

For the detection of HIF1α, the protocol was adjusted in order to further reduce background staining. Therefore, TBS and TBST (details in chapter 10.1) was used instead of PBS or PBST, which seemed to work better for the anti-HIF1α antibody.

The band intensity was read using the image analysis software (Image Lab, BioRad), allowing a semi-quantitative evaluation of protein expression. The SOD-values (Standardized Optical Density) used for the further statistical analyses were calculated by determining the arhythmical ratio of the optical density of the target protein and the reference gene B-ACTIN.
The calculations and the graphical visualization were done using Excel program (Microsoft, Redmond, WA, USA).

Table 3: List of antibodies used for Western Blot analysis

Antigen	Product Number	Producer/Company	Dilution	Species/type
STAR	-	Dr. DM Stocco, Texas Tech University Health Science Center Lubbock, TX, USA (Clark, Wells et al. 1994)	1:5000	Rabbit, polyclonal
CBP (D6C5)	7389	Cell Signaling Technology, Dallay, Texas, USA purchased from Bioconcept	1:1000	Rabbit, monoclonal
CREB (48H2)	9197	Cell Signaling Technology	1:1000	Rabbit, monoclonal
cJUN (60A8)	9165	Cell Signaling Technology	1:1000	Rabbit, monoclonal
HA-Tag (C29F4)	3724	Cell Signaling Technology	1:1000	Rabbit, monoclonal
B-ACTIN (ACTB D11B7)	sc81178	Santa Cruz Biotechnology, Inc., Dallas, TX, USA	1:2000	Mouse, monoclonal
HRP-labeled anti rabbit IgG	31821	Thermo Fisher Scientific, Pierce Biotechnology, Rockford, IL, USA	1:15000	Donkey, polyclonal
HRP-labeled anti mouse IgG	W4021	Promega, Dübendorf, CH	1:15000	Goat, polyclonal

5.3 Semi-quantitative real time (TaqMan) polymerase chain reaction (PCR)

5.3.1 RNA-Isolation

Total RNA was isolated using TRIzol® reagent (Ambion, Life Technologies, Thermo Fisher Scientific AG) according to manufactures instructions. Thus, after washing with ice-cold PBS, cells were collected by adding 500µl of TRIzol® directly to the culture plate. After 10min incubation on a shaker, the cell lysates were transferred to a 1.5ml Eppendorf tube and were kept on ice. Next, 100µl of chloroform (Roth GmbH & Co., Arlesheim, CH) were added and the tube was mixed on Vortex. After 5min incubation time on ice, the samples were centrifuged for 20min at 20'000x g at 4°C. Then, the aqueous RNA layer was transferred into a new Eppendorf tube, whereas the rest was discarded. The RNA was purified repeating the step with chloroform. Next, equal amounts of ice-cold isopropanol (2-propanol, Sigma Aldrich Chemie GmbH) were added and the tube was placed for 30min at -20°C. For pelleting the precipitated RNA tubes were centrifuged for 10min at 20'000x g at 4°C. The pelleted RNA was then washed twice by adding 500µl 70% ethanol (Roth GmbH & Co.) and centrifuging for 10min at 20'000x g at 4°C. The excess ethanol was removed and the pellet was dried for approx. 10min at ambient temperature. Next, the pellet was re-suspended in 20µl sterile double distilled water (ddH_2O, bidest. water) and incubated for 10min at 50°C in a Thermomixer (Thermomixer comfort,

Vaudaux-Eppendorf AG, Basel, CH). Subsequently, 1µl of RNase-Inhibitor (Riboloc, RNase Inhibitor, 40U/µl, Fermentas, Le-Mont-sur-Lausanne, CH) was added. The RNA content was checked with a NanoDrop 200C® spectrophotometer (Thermo Fisher Scientific AG). Based on the received RNA concentrations a working solution was generated by diluting the RNA with sterile ddH_2O to a final concentration of 200ng/µl. The working and stock solutions were kept at -80°C until further analysis.

5.3.2 DNase treatment

In order to remove any potential contamination with genomic DNA, in the next step, DNase treatment with RQ1 RNase DNase (Promega) was performed for each sample following the manufactures instructions. The mix is presented in Table 4. It was then incubated for 30min at 37°C in Thermocycler (Mastercycler, Vaudaux-Eppendorf AG). Immediately afterwards 1µl of DNase-stop-solution was added to each sample followed by 10min incubation at 65°C for deactivation of DNase.

Immediately after the DNase treatment, the RT (reverse transcription) reaction was performed.

Table 4: DNase treatment master mix

Reagent	**Volume (µl)**
RQ1 DNase Buffer	1
RQ1 DNase RNase-free	1
Sterile ddH2O	1.25
RNA working solution	6.65
Total volume	**9.9**

5.3.3 Reverse transcription (RT)

The RT reaction enables the synthesis of complementary (c)DNA from the isolated total RNA, which is needed for the further analysis by real time PCR. The RT was performed with reagents purchased from applied Biosystems by Thermo Fisher Scientific AG and was carried out following manufactures instructions and as previously reported protocol (Kowalewski, Schuler et al. 2006). Random hexamers served as primers and 1.5µl of the DNase-treated RNA solution was used per each RT reaction.

Table 5: RT master mix

Reagent	**Volume (µl)**
Sterile ddH2O	1
RT-Buffer 10x	2
$MgCl_2$ 25mM	2
dNTP mix 10nM	2
Random hexamers 50µM	0.5
RNAse inhibitor 20U/µl	0.5
Reverse transcriptase (AMV-RT) 50U/µl	0.5
Total Volume:	**8.5**

The RT reactions were carried out in an Eppendorf Mastercycler (Vaudaux-Eppendorf AG, Basel, CH) under the following conditions:

Table 6: Phases of RT reaction

Phase	Condition
1	8min 21°C
2	15min 42°C
3	5min 99°C
4	hold 4°C

5.3.4 Semi-quantitative real time (TaqMan) polymerase chain reaction (PCR)

Real time (TaqMan) PCR is a well-established tool in molecular biology and allows the detection of the PCR product simultaneously during amplification time. With this, the gene expression of a target gene can be easily quantified. The amplification of nucleic acids requires specific primers, similarly to the classical PCR, with an addition of a fluorescent (TaqMan) probe. The latter is an oligonucleotide labelled with a reporter dye (6-carboxyfluorescein, acronym: FAM), covalently bound to the 5'end, and a quencher (6-carboxytetramethyl-rhodamin, acronym: TAMRA) attached to the 3'end. The quencher inhibits any fluorescence signals from FAM as long as both are in a close proximity. The probe anneals to a complementary cDNA region. When, following the cDNA synthesis along the template, Taq-polymerase reaches the TaqMan probe, due to its 5'-3'-exonuclease activity, the Taq-polymerase degrades the probe annealed to the template. The degradation of the probe results in the release of the fluorophore, the light of which is measured by the quantitative PCR thermal cycler. In this way, with each cycle of PCR more dye molecules are released, resulting in an increase in fluorescence intensity. The fluorescence intensity detected is proportional to the number of synthesized amplicons. Importantly, a signal can be only detected if the target cDNA is available and suitable primers were used.

Here, the TaqMan PCR reactions were carried out in an automated fluorometer ABI PRISM 7500 Sequence Detection System (Applied Biosystems by Thermo Fisher Scientific AG) using Fast Start Universal Probe Master (ROX) (Roche, Basel, CH). Each sample was analysed in duplicate and as negative controls autoclaved ddH_2O and the so-called RT-minus control were used instead of cDNA. This RT-minus control corresponds to samples, which were run omitting reverse transcription and therefore reassure that there was no contamination with genomic DNA, and additionally prove the accuracy of the DNase treatment. The reactions were performed using 96-well optical plates (Applied Biosystems by Thermo Fisher Scientific AG).

The reaction mixtures of 10µl were prepared as follows: 6.25µl Fast Start Probe Master (ROX), 0.5µl TaqMan probe (200nM), 0.75µl forward primer (300nM) and 0.75µl reverse primer (300nM). This 10µl of reaction mixture was put into each well and 2.5µl of cDNA from the reverse transcription were added to a get a total reaction volume of 12.5µl. The amplification conditions were set up as follows: initial denaturation of 10min at 95°C followed by 40 cycles at 95°C for 15sec and 60°C for 60sec. All reactions were run in duplicates.

In-house designed primers and probes for *Star* and *Gapdh* were prepared using Primer Express software version 2.0 (Applied Biosystems by Thermo Fisher Scientific AG) and purchased from Microsynth, Balgach, CH. The list of TaqMan systems is presented in Table 7.

Table 7: List of primers and TaqMan systems used for real-time (TaqMan) PCR

Gene	**Accession numbers**	**TaqMan System**	**Product length (bp)**
Gapdh	AB028142	*Forward:* 5'-GCA GTG AAG GGA TGG AGA TTG-3' *Reverse:* 5'-GTG AGT GGA GTC ATA CTG GAA CAT G-3' *TaqMan Probe:* 5'-TCA ACG ACC CCT TCA TTG ACC TC-3'	75
Star	EF522840	*Forward*: 5'-CCG GGT GGA TGG GTC AA-3' *Reverse*: 5'-CAC CTC TCC CTG CTG GAT GTA-3' *TaqMan Probe*: 5'-CGA CGT CGG AGC TCT CTG CTT GG-3'	65
Actinb		Pre-designed assay from Applied Biosystems, Prod. No. Mm01205647_g1	72
cJun		Pre-designed assay from Applied Biosystems, Prod. No. Mm00495062_s1	81
Crebbp		Pre-designed assay from Applied Biosystems, Prod. No. Mm01342430_m1	100

5.3.5 Relative quantification of target gene expression

The relative quantification of target gene expression was obtained by using two reference genes (*Gapdh* and *Actinb)* in the comparative CT method (ΔΔCt method). The analysis was performed according to the directions of the manufacturer (ABI PRISM® 7500 fluorometer) and to the protocol described previously (Kowalewski, Meyer et al. 2011). The efficiency of the PCR assay was established using the CT slope method to ensure approximately 100% reaction efficiency.

In the ΔΔCt method, the threshold cycle (Ct-Value) is calculated at the beginning. The Ct value corresponds to the PCR cycle in which the fluorescence intensity of the degraded TaqMan probe reaches above background fluorescence. At this point the amplification of the cDNA occurs exponentially. The Ct values are inverse to the amount of cDNA in the sample, that means the lower the Ct value the higher the number of target gene copies in the sample, because less cycles are needed to reach the threshold cycle. To be certain that the variation in the Ct values are due to real biological changes, the Ct values are compared to the Ct values obtained for the reference gene/genes. Reference genes are supposed to be equally expressed and are should be not subjected to any experimental fluctuations.

This comparison is calculated as follows:

$$\Delta Ct_{sample} = \Delta Ct_{target\ gene} - \Delta Ct_{reference\ gene}$$

The ΔCt of the target gene and reference gene corresponds to the arithmetical mean received from the measured duplicates.

In the next step, the obtained ΔCt value for each sample is normalized to the calibrator (sample with highest Ct value, i.e. lowest amount of respective target gene transcript) in order to receive the ΔΔCt-value. The following formula was used:

$$\Delta Ct_{sample} - \Delta Ct_{calibrator} = \Delta\Delta Ct$$

Finally, the relative gene expression (RGE) is calculated for graphical illustration:

$$RGE = 2^{(-\Delta\Delta Ct)}$$

The RGE corresponds to the n-fold overexpression of the target gene relatively to the calibrator.

5.4 Expression vectors

5.4.1 PRc/RSV-m CBP-HA expression vector

For the establishment of a reliable detection system for CBP in KK1 granulosa cells by Western Blot analysis, overexpression approach was chosen. Therefore, a commercially available pRc/RSV-m CBP-Hemagglutinin (HA) expression vector was selected and purchased from Addgene, Watertown, MA, USA (Addgene plasmid #16701; http://n2t.net/addgene:16701; RRID: Addgene_16701, see Figure 5). This vector consists of a pRc/RSV-m vector backbone and a mouse CBP gene insert (Gen Bank ID: NP_001020603.1) fused with a Hemagglutinin (HA) protein tag. The pRc/RSV-m vector backbone has a length of 5.2kb and shows high level of stable expression in eukaryotic cells. It carries a RSV LTR (Rous sarcoma virus terminal repeat) promoter and enhancer expressing inserted genes in eukaryotes, a multiple cloning site (MCS, polylinker), a SV40 origin and a neomycin resistance marker, which allows a selection with G418 (geneticin). For growth and maintenance in DH5α bacteria it has an ampicillin resistance marker and an origin of replication. The CBP-HA gene insert has a size of 8kb. The HA protein tag is fused to the C-terminal site of the gene insert and facilitates the detection, isolation and purification of CBP. For cloning the vector has a HindIII 5'cloning site and a NotI 3'cloning site.

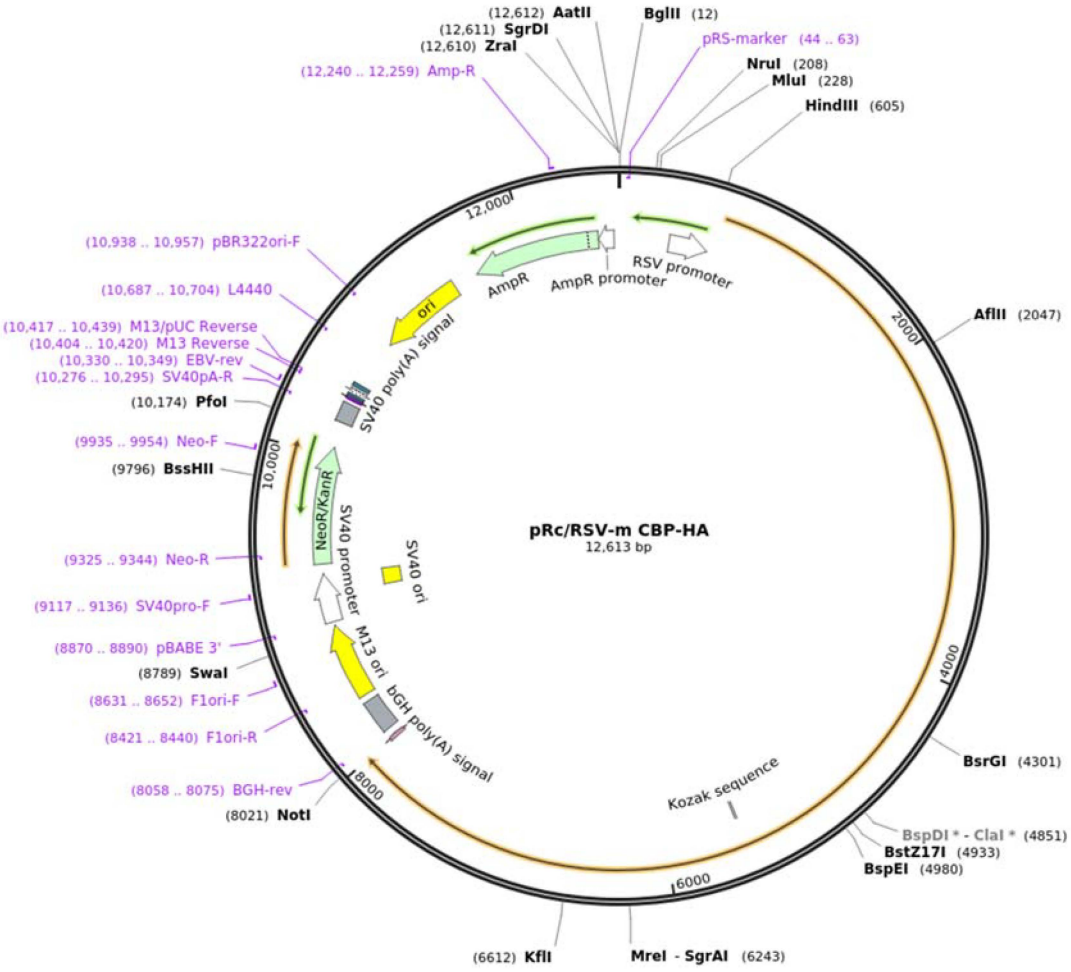


Figure 5: Addgene Full Sequence Map for pRc/RSV-m CBP-HA.

5.4.2 Flag-cJUNWT-Myc expression vector

The Flag-JUNWT-Myc expression vector (Addgene plasmid #47443; http://n2t.net/addgene:47443; RRID: Addgene_47443, see Figure 6) was used as an additional control vector to carry out immunoprecipitation experiments. It has a total size of 5342bp, which makes transfection easier and increases its efficiency. Furthermore, the cJUN antibody showed low background staining (see master thesis (Lanfranchi 2016)), facilitating the detection of cJUN in Western Blot analysis. The vector backbone is a pCMV-Tag2A vector with a size of 4300bp. The promoter of the vector backbone is a CMV (Cytomegalovirus) having high expression in mammals and positive clones can be selected using neomycin. The Flag-JUNWT-Myc plasmid growths in DH5alpha bacteria and kanamycin can be used as a resistance marker. The gene insert carries the mouse JUN sequence (Gene Bank ID: 16476) and has a size of 1042bp. The insert is tagged with a C-terminal Myc protein, whereas the backbone has a N-terminal Flag fusion protein.

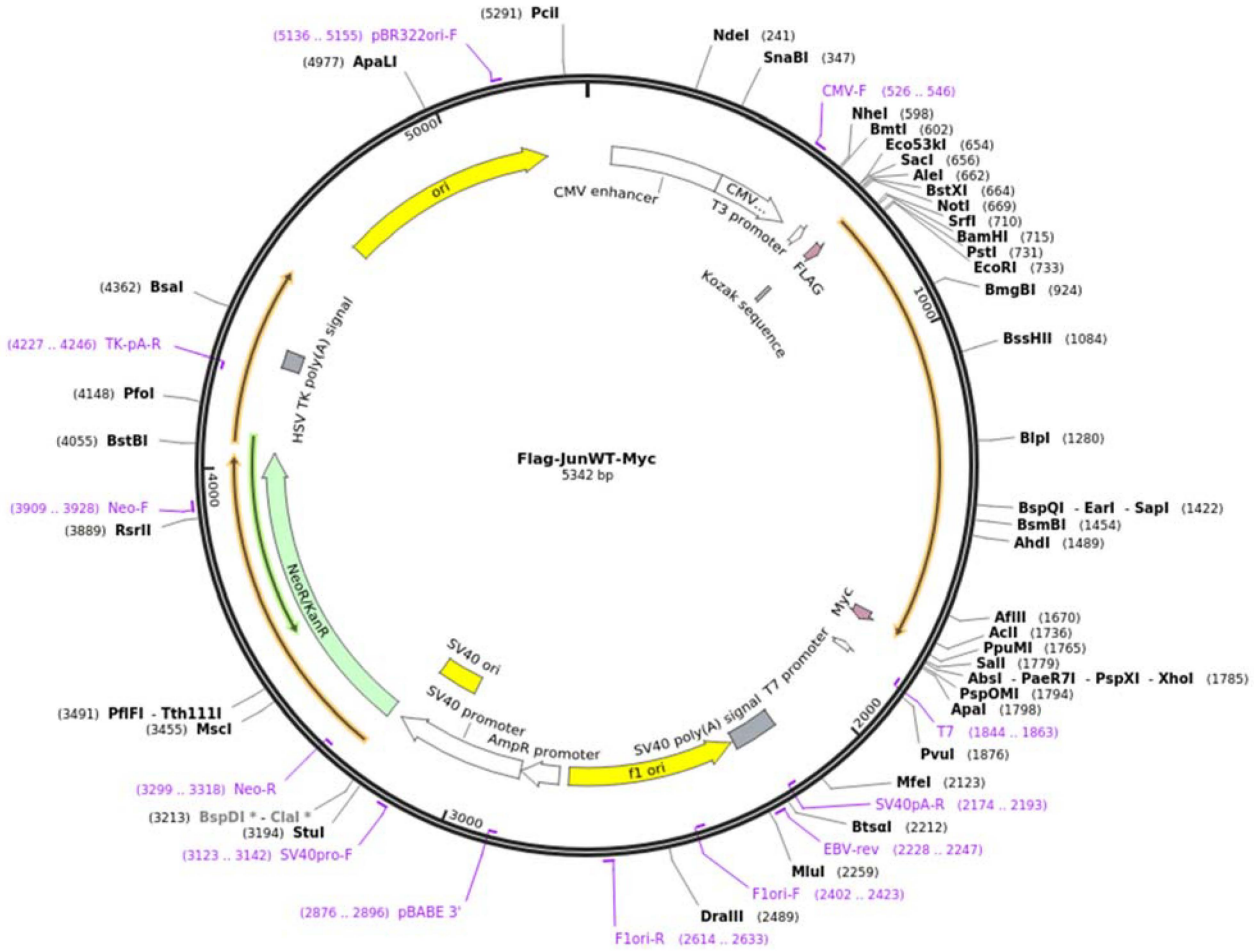


Figure 6: Addgene Full Sequence Map for Flag-JUNWT-Myc.

5.4.3 Control plasmid - Assembling of pRc/RSV-MCS vector

The pRc/RSV-MCS (empty) vector was used as a negative control for transfection experiments. The control plasmid ensures that the observed phenomenon is associated with the overexpression of target protein itself and not by any unintended variable induced by the transfection. The empty vector was created by replacing the CBP-HA gene insert, flanked by HindIII and NotI recognition sites, with the original multiple cloning site (MCS, Polylinker) of the backbone vector. Therefore, MCS polynucleotide flanked by HindIII and NotI was used (Figure 7*)*.

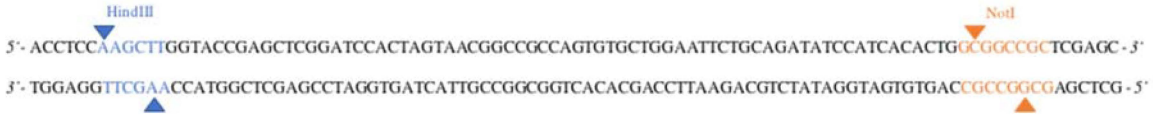


Figure 7: Sequence of polylinker (MCS, multi cloning site) with marked recognitions sites for NotI and HindIII.

Annealing of oligonucleotides to create dsDNA

In the first step, polynucleotides (i.e., MCS forward and reverse sequence) were annealed in the annealing buffer (see details in chapter 10.1). Therefore, 10µl from each strand (100µM stock, forward or reverse) were added to an Eppendorf tube and mixed with 80µl of annealing buffer to reach a final concentration of 10µM. Samples were heated for 15min at 95°C and subsequently left for 45min at ambient temperature.

Restriction digestion

In order to insert the MCS into backbone, sticky ends need to be created that are complement with the overhangs of the vector. Therefore, the vector and the MCS insert were both digested with the restriction enzymes NotI and HindIII.

Table 8: Protocol for master mix for restriction digestion

Reagents	**Volume (µl)**	**Annealed polynucleotides**
NEBuffer™3.1*	5	4
NOT I*	5	2
HIND III*	10	4
DNA	20 (10µg)	8 (4µg)
ddH2O (ad to 50µl)	10	22
Total Volume:	**50**	**40**

- * Double amount of HindIII was used, as the enzyme shows only 50% efficiency in NEBuffer™3.1; reagents were purchased from New England Biolabs Inc, Ipswich, MA, USA.

The restriction digestion was done for 2h at 37°C. The digested plasmid was loaded on 1.5%, whereas the polynucleotides on 2% agarose gel with ethidium bromide. The DNA was purified from gel using Qiaex II gel extraction system (Qiagen AG, Hombrechtikon, CH). The

concentration of extracted DNA was measured with NanoDrop 200C® spectophotometer (Thermo Fisher Scientific AG).

Ligation

Table 9: Protocol for ligation

Reagents	**Volume (µl)**
2x Rapid Ligation Buffer, T_4 DNA Ligase*	4
Vector	2
Insert (MCS)	3
T4 Ligase, 3U/ µl*	1
Total Volume	**10**

* components were purchased from Promega

The Ligation of the vector backbone and the polylinker was performed overnight at 4°C.

Transformation of competent E. coli (Escherichia coli) bacteria (XL1-Blue type)

The XL1-Blue type bacteria were purchased from Stratagene, La Jolla, CA, USA). To transform the bacteria with the ligated plasmid DNA of the pRc/RSV-MCS vector, the bacteria were thawed carefully on ice. The transformation protocol was performed according to the manufacturers protocol and included the so-called heat shock procedure. Thus, the ligation product was added to the bacteria suspension and was exposed to a heat pulse of 45sec (critical for maximal transformation efficiency). The bacteria were then propagated in LB Medium (DificoTM LB Broth, Miller (Luria-Bertani), Bectani, Becton, Dickinson and Company, Spars, USA), before plated on Agar plates (DificoTM Agar, Miller (Luria-Bertani), Bectani) with ampilicin (100µg/ml, Sigma Aldrich Chemie GmbH) and kept overnight at 37°C. The next day colonies were picked and cultured overnight at 37°C in 3ml LB medium in the presence of ampilicin (100µg/ml).

Plasmid-isolation and control restriction digestion

Propagated plasmids were isolated with the Qiagen Plasmid Maxi Kit (Qiagen AG) following manufactures instructions. The concentration of the isolated DNA was measured with NanoDrop 200C® spectophotometer (Thermo Fisher Scientific AG).

Thus, the plasmid was digested with the restriction enzymes NotI and HindIII for 2h at 37°C and loaded on 1.5% agarose gel with ethidium bromide. Thereby, the success of the ligation procedure was verified.

Table 10: Restriction digestion protocol

Reagents	**pRc/RSV-m CBP-HA**	**pRc/RSV-m MCS**
NEBuffer™ 3.1	2.5µl	2.5µl
NotI	2.5µl	2.5µl
HindIII	5µl	5µl
Plasmid (2 µg)		
ddH2O **add 25 µl**		

5.5 Transient transfection of KK1 cells

KK1 cells were transiently transfected with either pRc/RSV-m CBP-HA, pRc/RSV-m MCS (empty vector) or with Flag-JUNWT-Myc vector. For transfection cells were seeded in 6-well plates (TPP®) the day before in order to reach 50-60% confluence. The transfection was carried out using FuGENE HD transfection reagent (Roche) and was performed following previously described protocol (Kowalewski, Dyson et al. 2009). In short: the ratio of FuGENE HD transfection reagent to DNA was 1:3.5, thus for 1µg of DNA 3.5µl of transfection reagent were applied. Each well was transfected with 100µl transfection mixture containing medium without any adds ("no-adds" medium), transfection reagent and 1µg of DNA per well.

Prior to transfection the "no-adds" medium was mixed with FuGENE HD reagent and incubated for 5min at ambient temperature. Subsequently, the DNA was added, and the mixture was left for 25min in order to generate DNA/reagent complexes. In the meantime, cells were washed with 1x PBS and 1ml of serum-containing normal culture medium was added to each well. After 25min, 100µl of transfection mixture were added and cells were placed back in the incubator. After 6h, cells were supplied with additional 2ml of normal culture medium. At the next day, the medium was replaced with 3ml of fresh complete culture medium and cells were placed into specific culture condition (normoxia (20% O_2) or reduced oxygenation (10% O_2)). The stimulation of the cells occurred 24h thereafter.

5.6 Transient transfection of HEK293T cells

Transient transfection of HEK293T cells with pRc/RSV-m CBP-HA and Flag-JunWT-Myc vectors was carried out using Calcium Phosphate (Ca/P) protocol. To control transfection efficiency, one plate was transfected with a pCMV-GFP vector only. The detected fluorescence at the day after transfection (72h) allowed an approximate estimation of transfection efficiency. The HEK cells were seeded on a 10cm culture plate the day before transfection to reach approx. 60% confluence on the day of transfection. Per culture plate 10µg plasmid (DNA) were used. The reaction mixture was prepared as follows: 430µl sterile ddH_2O, 50µl $CaCl_2$ (2.5M) and 10µg plasmid DNA were mixed in a 2ml Eppendorf tube. While vortexing the tube, 500µl BES (N,N-Bis-(2-Hydroxyethyl)-2-Aminoethane Sulfonic Acid) – buffered solution was added, and the tube was vortexed for further 10sec. The Eppendorf tube was then allowed to stand for 10sec followed by another 10sec of vortexing. The reaction mixture was then pipetted dropwise on the HEK293T cells. On the next morning, the whole media was replaced with fresh DMEM/Ham's F12 supplemented with 10% FBS and 1% penicillin/streptomycin (all purchased from Gibco). After 72h of transfection cells were harvested for further analysis.

5.7 Immunoprecipitation (IP) and co-Immunoprecipitation (co-IP)

For examination of the HIF1-mediated effects on CBP protein in KK1 granulosa cells, immunoprecipitation (IP) approach was chosen. In this thesis, three different IP approaches were tested and the developed protocols are described in the following chapter. The elaborated IP protocols included magnetic beads, agarose beads and protein-tagged beads (magnetic and agarose) as insoluble support. The procedures with magnetic and agarose beads were carried out in KK1 cells, whereas the method with protein-tagged beads was conducted in HEK cells used as a cellular model known for easier transfection properties. All protocols were elaborated independently. They were adapted to the objective of the experiment, the IP input and the cell line and insoluble support utilised. This required adjustment in incubation times, compositions of buffers and lysis solutions, as well as washing conditions.

Of the three protocols that were tested, the one developed for HEK cells proved to be the most suitable and resulted in presented data.

5.7.1 Principle

The IP method uses the antigen-antibody (ag-Ab) interaction principle to identify a protein of interest in a mixture. It contains the following steps:

1) The antibody against the protein of interest is pre-immobilized onto an insoluble support, either agarose or magnetic beads. This occurs through the interaction between protein A or G covering the surface of agarose or magnetic beads and the Fc-region of the antibody.
2) The antibody-agarose/magnetic beads conjugate is incubated with the cell lysate containing the target protein. During incubation time the immobilized antibody binds the target antigen forming an immobilized immune complex (IP step).
3) This complex is then captured, eluted from the support and analysed by SDS-Page and immunoblotting.

Alternatively, the antibody is directly added to cell lysate and allowed to form immune complexes with the target antigen (free antibody approach). These complexes are then retrieved by agarose or magnetic beads. Although the pre-immobilized antibody approach is more commonly used, the free antibody method can be beneficial if the target protein is present in low concentrations, the antibody affinity is weak or the binding kinetics of antigen to the antibody are slow. Additionally, there are specific magnetic or agarose beads coated, already with an antibody against common available Tag/fusion proteins, such as HA, GST or Flag Tag. This can be advantageous, if no suitable target antibodies are available.

In order to further analyse possible protein-protein interactions (existence of protein complexes), co-IP approach can be applied. In co-IP, an antibody is used to purify its target antigen (similarly to IP). If the target antigen is a part of protein complexes, further proteins or molecules can be detected by Western Blot analysis. This thesis reports first, still preliminary results from co-IP trials.

5.7.2 Preparation of protein homogenates in KK1 cells

The protein homogenates used for (co-)IP experiments were prepared as follows: Following stimulation experiments, cells were treated with a final concentration of 1% formaldehyde for 10min at 37°C to cross-link the DNA with its associated proteins. Then, the medium was removed and cells were washed twice with ice cold PBS. Cells were lysed by adding 70-100µl of NET-2 lysis buffer with protease inhibitor (10µl/ml, Sigma Aldrich Chemie GmbH), incubated on shaker for 10min, scraped and collected in an Eppendorf tube. Samples were placed subsequently on ice. Cell lysates were homogenized by sonication applying 2-3 cycles of 75Watt (40% efficiency) for 15sec on ice. Afterwards protein lysates were centrifuged for 10min at 10'000x g at 4°C, removing excess cell debris. The supernatant was transferred to fresh Eppendorf tube, whereas the pellet was discarded. Next, protein concentrations were measured as described above (see 5.2) by Bradford assay. The protein homogenates were divided into: 1) input control and 2) working solutions for IP. Immunoglobulin (non-immunized normal rabbit IgG (Cell Signalling Technology, Prod, no. 2729, purchased from Bioconcept) and no-antibody-treated group, served as negative controls and were taken from the non-treated samples. The protein homogenates for input control were prepared as described above for Western Blot analysis (see 5.2), by adjusting the concentration to 1.2µg/µl by adding NET-2 lysis buffer and 4x SDS sample buffer. The input control was heated for 10min at 95°C before loading on gel. The working solutions for IP groups were diluted with NET-2 lysis buffer in order to reach the highest possible and similar concentrations in all groups. Any left protein homogenates were stored as stock solution at -80°C.

5.7.3 Magnetic beads method

For IP with magnetic beads, Dynabeads^{TM} protein G kit (Thermo Fisher Scientific AG) was used. Dynabeads^{TM} protein G are homogenous supermagnetic beads of a size of 2.8µm. On their surface recombinant Protein G (approximately 17kDa) is covalently bound. The IP was carried out following manufactures instructions with small adjustments. Additionally, a preclearing step was included to reduce non-specific and background staining. The IP included the following steps presented in Table 11.

Table 11: IP protocol for magnetic beads

Step	Procedure
Preparation of protein homogenates	See 5.7.2
Preparation of Dynabeads™ (magnetic beads)	- resuspend magnetic beads and transfer 50µl into an Eppendorf tube - place on magnet (12 tube magnet, Qiagen AG) - remove supernatant
Pre-clearing	- add 200µl of working solution for IP to prepared magnetic beads - incubate with rotation for 1h at 4°C - place tube on magnet - transfer supernatant to a fresh tube - discard magnetic beads with unspecific antibody (Ab) bound
Antibody binding to Dynabeads™	- add 5µg of Ab (approx. 4µl) to 200µl Ab-binding and Washing Buffer (available in the kit) - pipette mixture to prepared magnetic beads - incubate with rotation for 1h at 4°C - place tube on magnet to pellet the Ab-conjugated magnetic beads - discard supernatant - wash Ab-conjugated magnetic beads 1x with 200µl of Ab-binding and Washing Buffer by gentle pipetting - put on magnet to remove supernatant
IP of target antigen	- add supernatant (antigen (ag) containing, from pre-clearing step) to pelleted Ab-conjugated magnetic beads, mix by gentle pipetting - incubate overnight with rotation at 4°C - pellet magnetic bead-Ab-ag complex by placing tube on magnet - wash 3x with 200µl Washing Buffer - resuspend Ab-ag-magnetic beads complex in 100µL Washing Buffer - transfer to a clean tube (to avoid co-elution of proteins bound to the tube wall)
Denaturating elution	- place tube on magnet and discard supernatant - add 20µl Elution Buffer (available in kit) and 10µl of 4x SDS sample buffer - heat for 10min at 95°C
SDS-PAGE /Immunoblotting	- put on magnet and load supernatant on gel - further steps see 5.2

5.7.4 Agarose beads method

IP experiments with agarose beads were performed under adjusted conditions using the reagents from Chromatin Immunoprecipitation Assay Kit (Merck Millipore, Upstate/Chemicon Temecula, CA, USA), following manufactures instructions. The following procedure was carried out presented in Table 12.

Table 12: IP protocol with agarose beads

Step	**Procedure**
Preparation of protein homogenates	See 5.7.2 - dilute sample (working solution for IP) 1:10 with Chip Dilution Buffer (available in kit) in a 2ml Eppendorf tube (take 200µl of working solution for IP) and add 1400µl of Chip Dilution Buffer)
Preclearing	- add 75µl of Salmon Sperm A Agarose 50% slurry to diluted sample - incubate for 1h at 4°C with rotation - pellet agarose beads with unspecific Ab bound for 1min 1000g - transfer supernatant to fresh Eppendorf tube, discard pellet
IP of target antigen	- add 4µg of specific Ab (approx. 4µl) to supernatant - incubate overnight with rotation at 4°C - add next day 20µl of Salmon Sperm/Protein A Agarose- 50% slurry - incubate for 1h with agitation at 4°C - pellet protein-Ab-agarose beads complex by gentle centrifugation (1min 1000g at 4°C)
Washing	- wash 5x 5min with 1ml NET-2 lysis buffer **OR** - wash 3x 5min with 1ml ice cold 1x PBS and 2x 5 min with TE Buffer (included in the kit). In between the washings, protein-Ab-agarose complexes were pelleted by gentle centrifugation and supernatant was discarded)
Denaturating elution	- add 20-30µl of 4x SDS sample buffer - heat for 10min at 95°C with 300rpm agitation - spin beads down for 1min at 1000g
SDS-Page /Immunoblotting	- load supernatant on gel - further steps see 5.2

5.7.5 Immunoprecipitation (IP) of CBP-HA and Flag-cJUN in HEK cells

The IP was carried out in HEK cells with the following steps presented in Table 13. The general protocols for Dynabeads and Agarose beads in KK1 cells described above (see 5.7.3 and 5.7.4) were modified and adjusted in order to have best IP output in HEK cells. For the immunoprecipitation of the overexpressed CBP-HA, anti-HA coated magnetic beads were used (Pierce Anti-HA magnetic beads, Thermo Fisher Scientific AG), whereas for the pull down of the Flag cJUN, ant-Flag agarose beads were applied (Anti-FLAG M2 agarose beads, Sigma Aldrich Chemie GmbH).

Table 13: IP protocol in HEK cells

Step	Procedure
Preparation of protein homogenates	- remove medium form cells and wash 1x with PBS - scrape lysate in 2ml PBS and transfer in Eppendorf tube - spin down at 800x g for 5min at 4°C - resuspend pellet in 2ml PBS and split in aliquots needed - spin aliquots 1000x g for 5min at 4°C - resuspend pellets in lysis buffer according to pellet size (approx. 100µl MNase buffer with freshly added protease inhibitor)
Incubation in lysis buffer	- add MNase (10U/5mill cells, for MNase digestion) - incubate for 30min at 37°C with rotation
Centrifugation	- spin down at 10'000x g for 5min at 4°C - keep supernatant for further steps (if desired, measure protein conc. with Bradford assay (see 6.2))
Split supernatant in aliquots	1) Input control (approx. 10% of total volume (20-30µl)) - add 4x SDS sample buffer - denaturate for 10min at 95°C continue with Western Blot analysis step or freeze in -80°C for later analysis 2) Working solution for IP (rest volume (70-80µl)) - dilute 4x in MNase buffer with protease inhibitor (approx. 280 – 320µl)
Immunopreciptation of target antigen	- add magnetic or agarose beads approx. 20-40µl (amount depending on protein concentration and manufactures recommendations); magnetic beads were washed before usage with MNase buffer (without protease inhibitor) 3x for 10min. - incubate overnight 4°C on orbit shaker
Collection of Ab-ag-beads complex	- pellet magnetic bead-Ab-ag complex by placing tube on magnet and - spin agarose bead-Ab-ag complex down for 1min at 0.6x g
Washing	- wash beads 3x for 10min with 1ml of C100 buffer
Denaturating elution	- resuspend beads in 30-40µl MNase buffer with protease inhibitor and add 4x SDS sample buffer - heat 5min at 95°C - put magnetic beads on magnet spin down agarose beads with max. speed (21'000xg) for 5min - load supernatant on gel
SDS-PAGE /Immunoblotting	- see 5.2 with following adjustments: Electrophoresis at 140V for 40min and transfer (Semi-Dry) for 2h at 18V followed by Ponceau staining

5.8 Statistics

Statistical analysis was carried out in cell culture experiments which were repeated at least for 3 times independently. The statistical software program GraphPad 3.06 (GraphPad Software, Inc., San Diego, CA, USA) was used and parametric one-way analysis of variance (ANOVA) followed by Turkey-Kramer Multiple Comparison was applied. The data is presented as the mean ± standard deviation (SD). In pilot experiments missing the minimal n-number of repeats, no statistical analysis was performed, and data are presented in a descriptive manner.

6 Results

6.1 Establishment of detection system for analysis of CBP protein expression in KK1 granulosa cells

6.1.1 Validation of anti-CBP antibody for Western Blot analysis

CBP is a large molecule with a molecular weight of 265kDa and the detection in Western Blot can be troublesome (Chrivia, Kwok et al. 1993). Thus, several commercially available anti-CBP antibodies were tested to assess the expression of CBP in KK1 granulosa cells, without, however reliable results *(data not shown)*.

Therefore, in order to validate antibodies and establish a reliable detection system for Western Blot analysis of CBP protein expression, an approach was chosen involving a CBP-HA expressing vector (pRc/RSV-m CBP-HA) (Chrivia, Kwok et al. 1993).

6.1.2 Synthesis of a pRc/RSV-m MCS (empty) vector

The transfection control for pRc/RSV-m CBP-HA was created in form of an empty vector. The empty vector was generated by substituting the CBP-HA gene insert with the original multiple cloning site of the vector's backbone (MCS, Polylinker). The oligonucleotides of the MCS were annealed forming double stranded DNA (dsDNA) and were digested with restriction enzymes NotI and HindIII. The digested DNA products were separated on 2% agarose gel (Figure 8 A). For ligation, the DNA was recovered by cleaning it from gel using Qiaex Kit.

Next, the pRc/RSV-m CBP-HA was enzymatically digested with NotI and HindIII and visualized on agarose gel (1.5%). Two bands were identified (Figure 8 B), indicating the 5.2kb plasmid backbone and the 8kb gene insert. The vector backbone was purified and prepared for ligation. Following ligation, the newly generated backbone plasmid (pRc/RSV-m MCS) was propagated in XL1-Blue competent E. coli bacteria.

The results of the control digestion with NotI and HindIII of the purified plasmid (pRc/RSV-m MCS) are presented in Figure 9. As control the original pRc/RSV-m CBP-HA was cleaved with the restriction enzymes NotI and HindIII and loaded simultaneously on agarose gel. This allowed the identification of the band of vector backbone in both, original and CBP-empty vector, whereas the band of the gene-insert was only present in original vector (Figure 9).

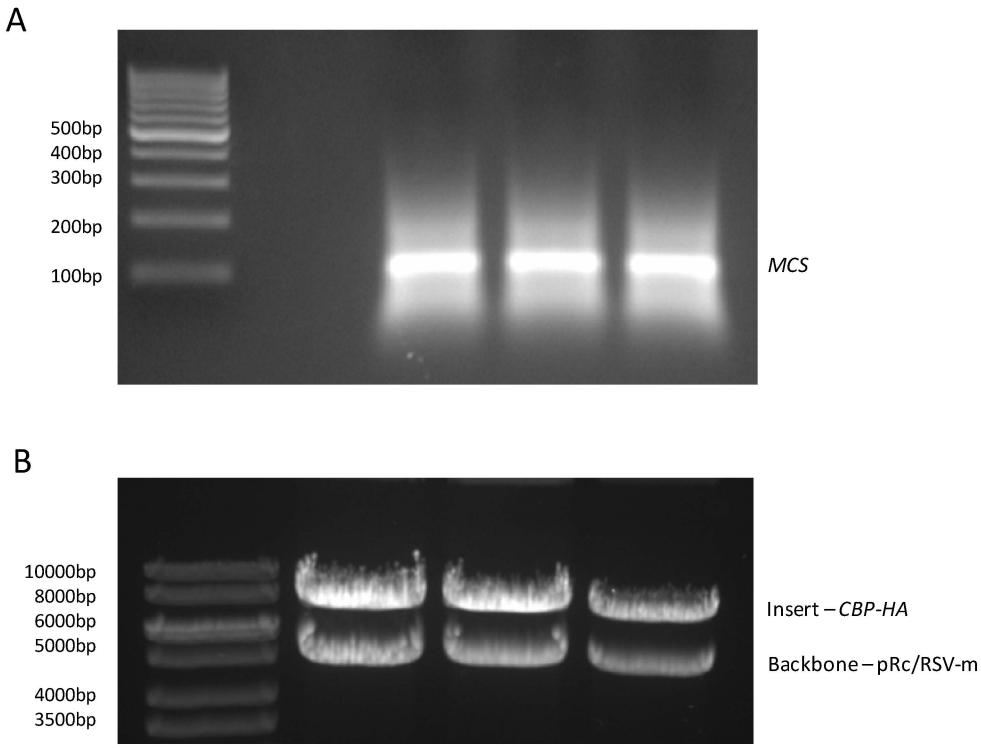


Figure 8: Preparation of pRc/RSV-m MCS vector. Presented are the DNA products after restriction digestion with the enzymes NotI and HindIII. The DNA ladder on the left indicates the relative DNA size. (A) Representative picture of DNA products separated on a 2% agarose gel after restriction digestion of polylinker (MCS). (B) Representative restriction products of the pRc/RSV-m CBP-HA vector (1.5% agarose gel). The vector backbone (5200bp) and the gene insert (8000bp) are visible.

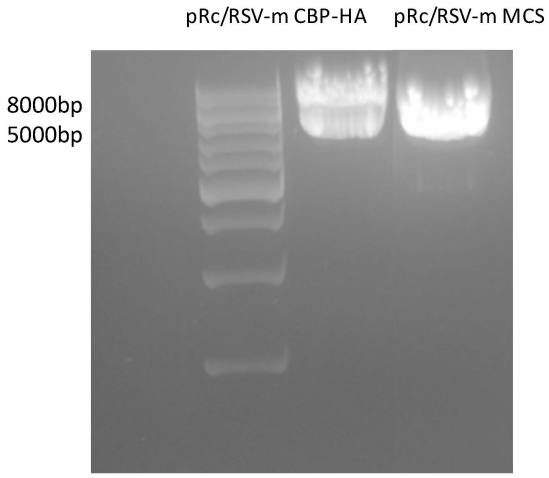


Figure 9: DNA products after control restriction digestion with NotI and HindIII. The relative DNA size is indicated with a DNA ladder on the left. Representative picture of cleaved plasmids pRc/RSV-m CBP-HA and pRc/RSV-m MCS are presented (1.5% agarose gel).

6.1.3 Overexpression of CBP in KK1 granulosa transfected with pRc/RSV-m CBP-HA and pRc/RSV-m MCS

The detection system for CBP was evaluated in KK1 cells under either normoxic (20% O_2) or under reduced oxygenated conditions (10% O_2). Cells were transiently transfected either with the CBP-HA expressing vector or with empty vector. KK1 cells previously transfected with each vector were treated with dbcAMP alone, or in combination with echinomycin for 6h. Non-treated cells served as controls. The expression of endogenous CBP and recombinantly expressed CBP-HA was determined by Western Blot with an anti-CBP and an anti-HA antibody. Representative pictures are shown in Figure 10 and 11 for experiments performed under 20% and 10% O_2, respectively. The anti-HA antibody detected recombinant CBP-HA protein, whereas the anti-CBP antibody detected both the endogenously expressed and recombinant CBP-HA (Figure 10 A and 11 A). Additionally, the semi-quantitative assessment of *CBP* (*Crebbp)* mRNA expression was performed by real time PCR (TaqMan) (Figure 10 B and 11 B). The expression of endogenous CBP appeared decreased in echinomycin-treated cells both, at the protein and mRNA level (Figure 10 and 11). The treatment with dbcAMP did not alter the levels of CBP.

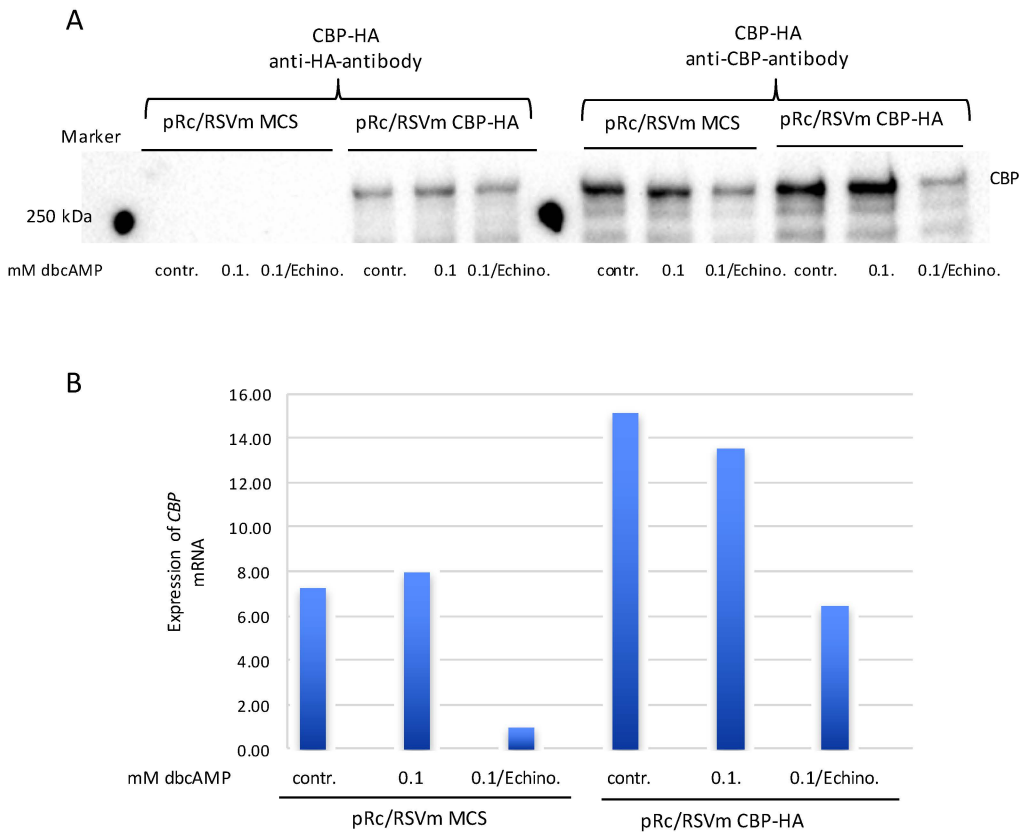


Figure 10: Assessment of CBP expression in KK1 granulosa cells under normoxic conditions (20% O_2). Cells were transiently transfected with pRc/RSV-m CBP-HA or pRc/RSV-m MCS expression vector. Transfected cells were then stimulated for 6h with or without 0.1mM dbcAMP in presence or absence of echinomycin (5nM) under 20% O_2. (A) Representative immunoblot is presented, performed with 25µg of protein lysate. The recombinant CBP-HA was recognized by the anti-HA antibody. The anti-CBP antibody detected the endogenously expressed and recombinant CBP-HA. (B) CBP mRNA expression by real time (TaqMan) PCR.

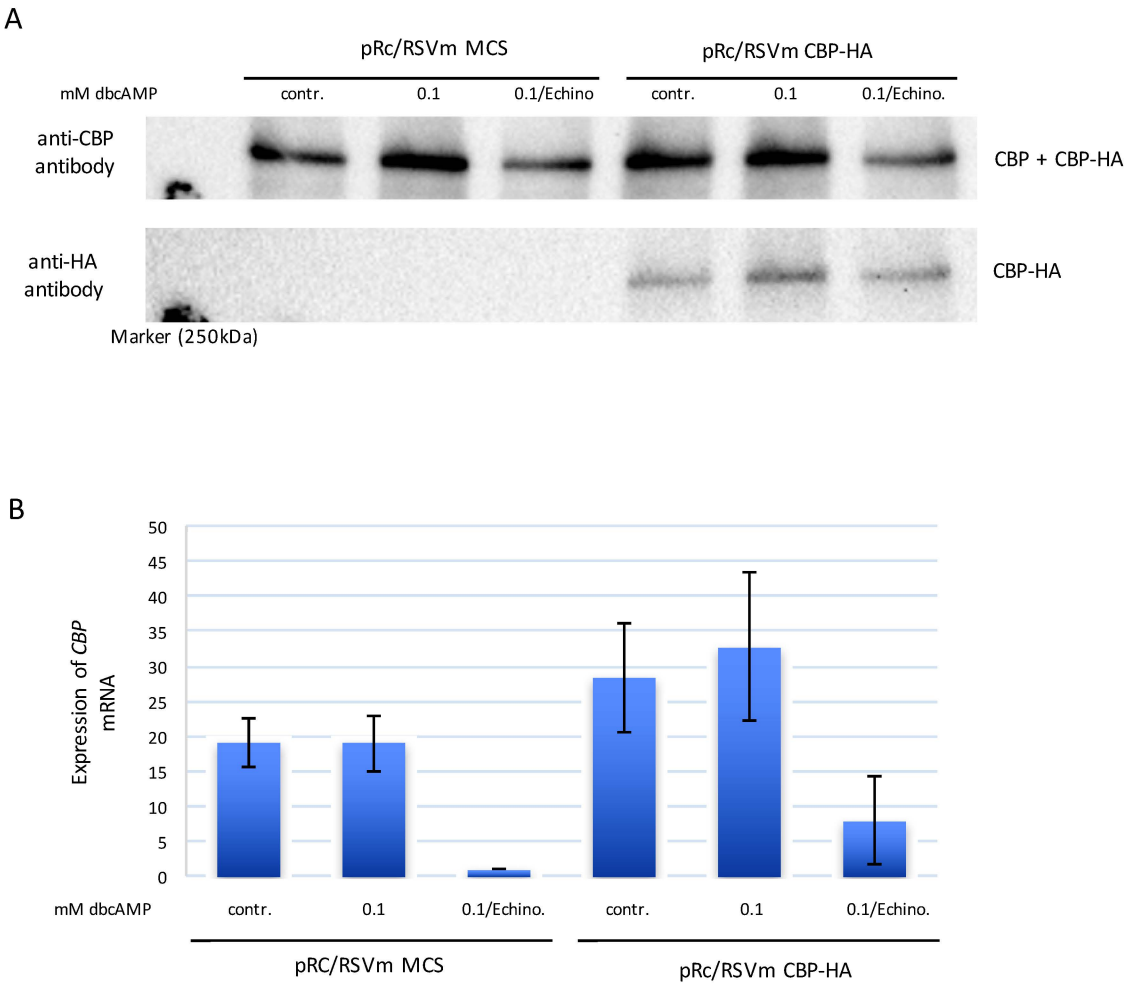


Figure 11: Evaluation of the expression of CBP in KK1 granulosa cells under reduced oxygenated conditions (10% O_2). Cells were transiently transfected with either pRc/RSV-m CBP-HA or pRc/RSV-m MCS expression vector and treated with 0.1mM dbcAMP in presence or absence of echinomycin (5nM) for 6h under 10% O_2. Non-stimulated cells served as control. (A) Representative immunoblot is shown. 25µg of protein lysate were used to perform the analysis. The anti-HA was used to detect the expression of recombinant CBP-HA, whereas the anti-CBP antibody visualized the endogenously expressed CBP and recombinant CBP-HA. (B) *CBP (Crebbp)* mRNA abundance assessed by real time (TaqMan) PCR.

6.2 Overexpression of cJUN in KK1 cells under reduced O_2 concentration (10% O_2)

In order to provide a basis for further investigation regarding the involvement of cJUN in HIF1-mediated steroidogenesis, cJUN overexpression experiments were established. Therefore, Flag-JUNWT-Myc expression vector was used.

The transfection protocol was validated under normoxic conditions (20% O_2). Following 48h of transfection, KK1 cells were stimulated with dbcAMP for 6h. Non-transfected cells served as control. Western Blot analysis was used to verify the cJUN expression, allowing for clear detection of the overexpressed protein (Figure 12 A). The mRNA levels of *cJun* were also determined (Figure 12 B). Treatment with dbcAMP did not affect the abundance of *cJun* mRNA and neither of the respective protein (Figure 12). Next, experiments were initialized aiming to assess the effects of cJUN overexpression in echinomycin treated KK1 cells under 10% O_2 (Figure 13).

As indicated from the presented pilot experiment (n = 1) (Figure 13) overexpression of cJUN appeared to overcome the diminishing effects echinomycin exerted on both, total and activated (P-) cJUN in control cells. Experiments presenting the STAR expression still need to be evaluated.

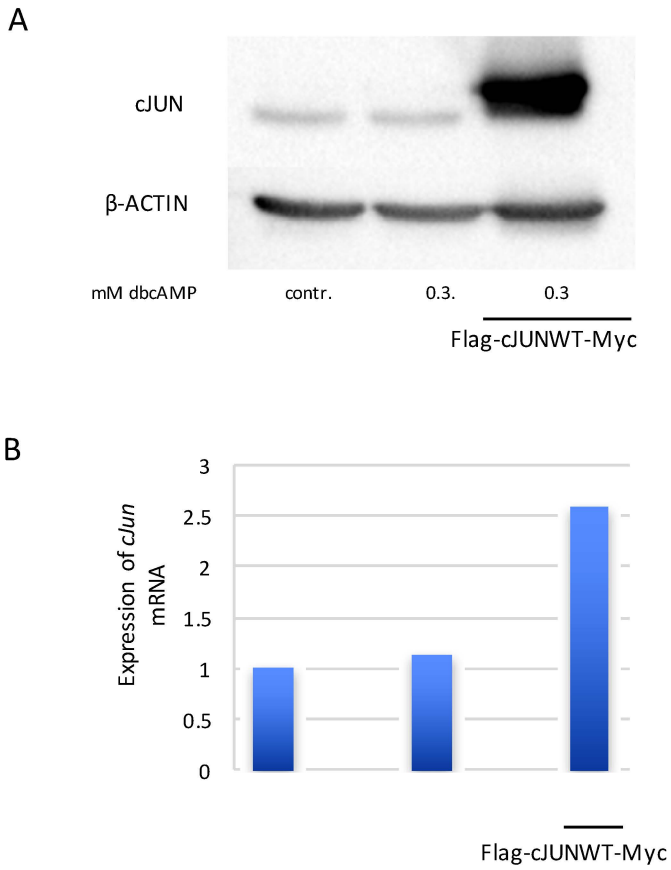


Figure 12: Establishment of transfection protocol for Flag-JUNWT-Myc expression vector. KK1 granulosa cells were transiently transfected with Flag-cJUNWT-Myc expression vector and stimulated for 6h in presence of 0.3mM dbcAMP under normoxic conditions (20% O_2) (n = 1). Non-transfected and non-treated cells served as control. (A) Western Blot analysis. cJUN (45kDa) expression was examined using anti-cJUN antibody. B-ACTIN (45kDa) was used as loading control. Immunoblot was carried out with 25µg of protein lysate. (B) Real time (TaqMan) PCR of *cJun* mRNA.

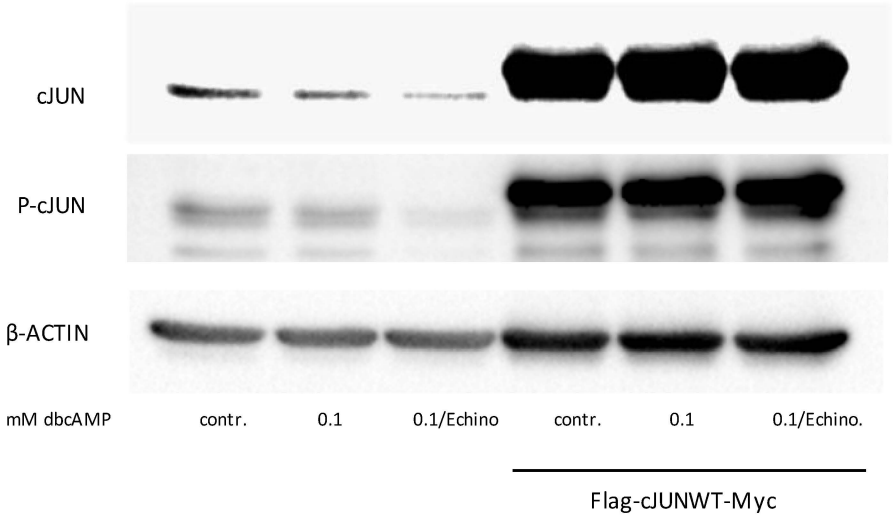


Figure 13: Effects of HIF1α activity on the activation of cJUN (P-cJUN) in KK1 cells expressing recombinant Flag-cJUNWT-Myc (n = 1). Cells were transiently transfected with Flag-JunWT-Myc and treated with or without 0.1mM dbcAMP for 6h under 10% O_2. The transcriptional activity of HIF1 was suppressed by echinomycin (5nM). Non-transfected cells served as control. Representative immunoblot detecting cJUN (45kDA), P-cJUN (45kDa) and B-ACTIN (45kDa, loading control) is presented. 20-30µg of protein lysate were used for analysis.

6.3 Validation of different immunoprecipitation (IP) protocols

With the aim to establish for future experiments a suitable IP protocol working with KK1 granulosa cells, different IP approaches were tested for detection of CBP and its associated proteins in larger complexes. The ultimate goal of these anticipated experiments is to assess the potential involvement of CBP in HIF1-mediated expression of STAR in KK1 granulosa cells under reduced oxygenated conditions (10% O_2).

The first tested IP protocol included immunoprecipitation with magnetic beads (Dynabeads™ Protein G kit) and salmon sperm DNA/Protein A agarose beads. Therefore, whole protein lysates from KK1 cells were used as IP-Input, which were transiently transfected with pRc/RSV-m CBP-HA and stimulated with dbcAMP over 6h in normoxic culture conditions (20% O_2). Several attempts and adaptions to the protocol were made in order to visualize the immunoprecipitated endogenous CBP and recombinantly expressed CBP-HA, as well as its possible binding partners CREB or HIF1α. However, the experiments did not produce reliable outcome *(data not presented)*. Large background staining made the interpretation of Western Blot analysis difficult producing non-specific bands mostly from the heavy and light chains of the denaturized antibodies used for the immunoprecipitation. Possible problems identified involved low IP-Input and possible too stringed washing steps. Therefore, the immunoprecipitation protocol was validated in HEK cells.

6.3.1 Establishment of immunoprecipitation protocol in HEK cells

To establish, the protocol for CBP-HA immunoprecipitation HEK cells represent an easier tool to transfect and are a good vehicle for the expression of recombinant proteins. HEK cells were transiently transfected with pRc/RSV-m CBP-HA expressing vector. The transfection experiment was carried out under normoxic culture conditions (20% O_2) and did not involve stimulation with dbcAMP. The transfection efficiency was controlled by measuring the fluorescence of a GFP vector 24h following transfection (*data not shown)*. After 48h of transfection, the recombinant CBP-HA was pulled down with HA-tagged magnetic beads and successfully visualized in Western Blot analysis with anti-CBP-antibody (Figure 14 A). Non-immunoprecipitated lysates served as an input control.

In parallel, cJUN protein was recombinantly expressed from Flag-cJUNWT-Myc vector in HEK cells under normoxic conditions (20% O_2). The recombinant cJUN was immunoprecipitated by means of anti-FLAG covered agarose beads. The detection of the purified target (Flag-cJUN) was identified by an antibody directed against cJUN (Figure 14 B).

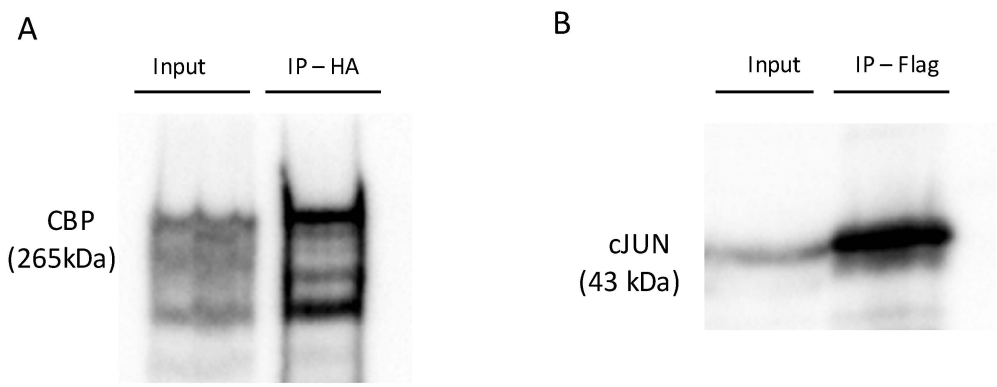


Figure 14: Immunoprecipitation of CBP-HA and Flag-cJUN in HEK cells (n = 1). (A) Immunoblot representing purified CBP from HEK cells transiently transfected with pRc/RSV-m CBP-HA expressing vector. Non-immunoprecipitated lysate served as input control. (B) Immunoprecipitated Flag-cJUN in HEK cells. Cells were transiently transfected with Flag-cJUNWT-myc expressing vector. The recombinantly expressed Flag-cJUN is visualized in Western Blot by an anti-cJUN antibody. The input control is indicated.

7 Additional experiments

The following section elaborates additional experiments performed in order to evaluate possible reduced oxygenation-mediated effects in testicular Leydig cell function. For this, the murine mLTC1 Leydig cell line was chosen as an appropriate cell culture model. The knowledge about the involvement of reduced oxygenation and HIF1α in STAR-mediated steroidogenesis in the testes and therefore in their physiological function is restricted. This is despite the fact that the testes are known to be physiologically exposed to low O_2 conditions (Palladino, Pirlamarla et al. 2011). These experiments aim to clarify if the investigated effects in granulosa cells are unique or if there are common regulatory features in steroidogenic cells in the gonads.

7.1 Characterization of the murine mLTC1 Leydig cells: Validation of STAR expression

The expression of STAR in immortalized mLTC1 Leydig cells was evaluated in preliminary experiments applying increasing dosages of dbcAMP (Figure 15). The role of PKA was verified by treatment with H89, a specific PKA activity inhibitor. All treatment experiments were performed in serum-free RPMI-1649 medium for 6h under normoxic conditions (20% O_2). Following stimulation, cells were collected and lysates were subjected to Western Blot analysis. Despite the high variation, the expression of STAR appeared to increase in dose-dependent manner and the treatment with H89 diminished the STAR levels (Figure 15). Nevertheless, due to the low n-number of experiments further analyses are needed for statistical verification.

Next, presented in Figure 16, the capability of mLTC1 Leydig cells to respond to the reduced oxygenated condition (10% O_2) was determined by analyzing the HIF1α expression by Western Blot. The mLTC Leydig cells were treated with dbcAMP under normoxic (20% O_2) and reduced O_2 conditions (10% O_2). Interestingly mLTC1 Leydig cells showed clearly detectable basal expression of HIF1α under 20% O_2, which despite an apparent increase, was not significantly elevated under 10% O_2 ($P = 0.1811$). The treatment with dbcAMP did not alter significantly ($P > 0.05$) the HIF1α abundance.

In the next step, the effect of reduced O_2 levels on STAR expression in mLTC1 cells were evaluated under 10% O_2 (Figure 17). The cells were stimulated with increasing dosages of dbcAMP with or without echinomycin and H89 over a period of 6h, either under 20% O_2 or 10% O_2. Non-treated cells served as control. Total STAR protein expression was assessed by Western Blot analysis and the abundance of *Star* mRNA was determined by semi-quantitative TaqMan PCR. The presence dbcAMP dose-dependently upregulated STAR expression at the mRNA and protein level in both, normoxic (20% O_2) and reduced O_2 conditions (10% O_2) (Figure 17). The STAR expression reached the highest level in response to 0.3mM dbcAMP and was significantly suppressed in the presence of echinomycin. The level of STAR protein stayed unaltered under reduced oxygenation (10% O_2) in mLTC1 Leydig cells (Figure 17).

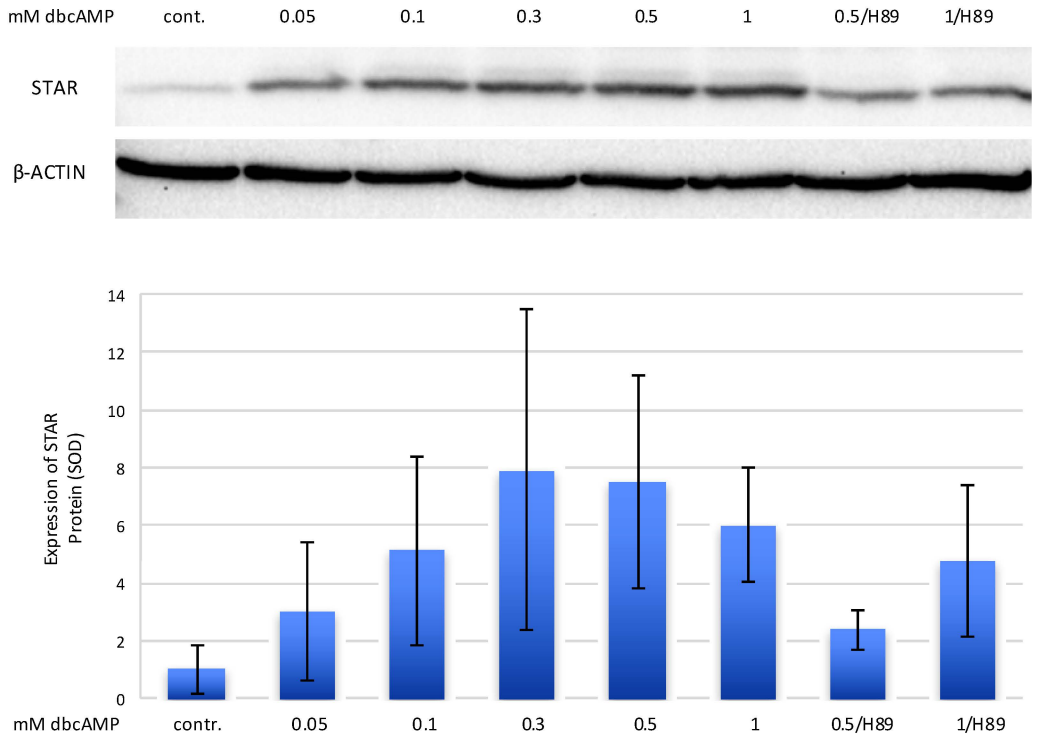


Figure 15: Preliminary result of the STAR protein expression in mLTC1 Leydig cells under 20% O_2. Cells were treated for 6h with increasing doses of dbcAMP (0.05-1mM) in serum free RPMI-1649 medium. The PKA activity was inhibited by applying H89 (25µM). A representative immunoblot is presented. The Western Blot analysis of STAR (30kDA) and B-ACTIN (45kDa) was done by using 25µg of protein lysate. The average standardized optical density (SOD) for STAR protein (mean ± SD) is shown.

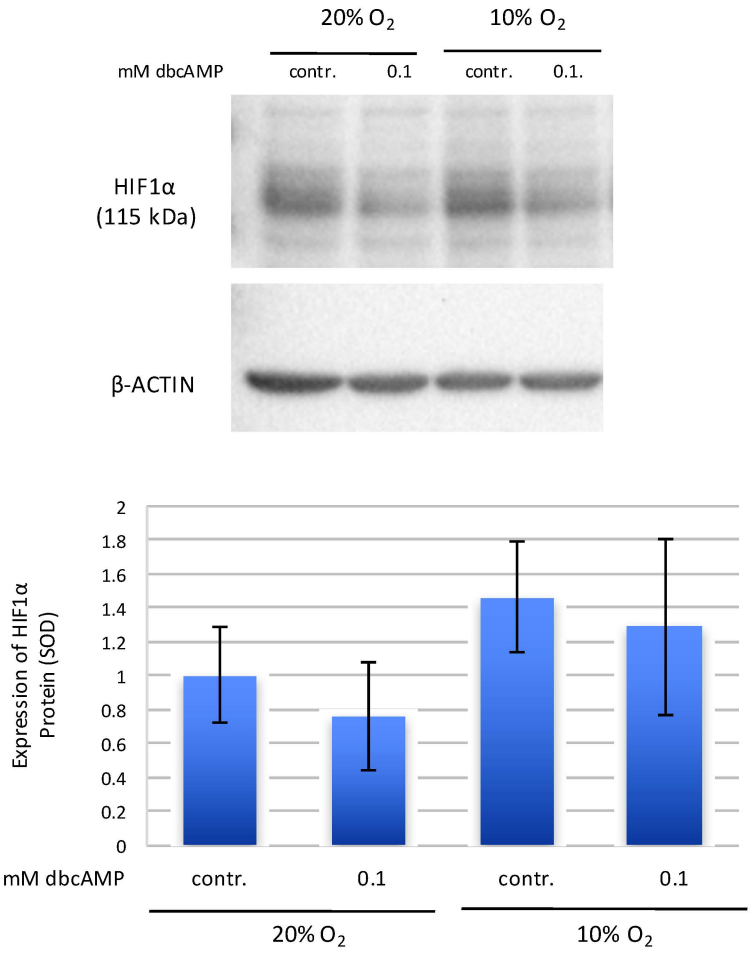
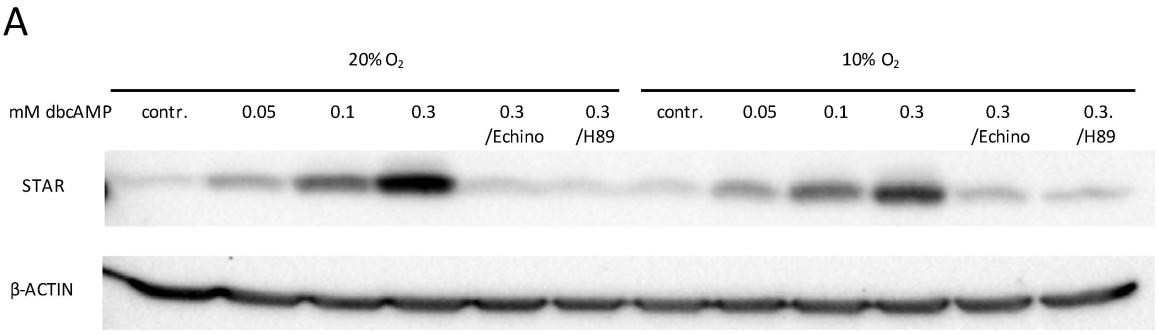


Figure 16: Expression of HIF1α under 20% and 10% O_2 in mLTC1 Leydig cells. Cells were cultured in serum free RMPI-1649 medium in presence or absence of 0.1mM dbcAMP for 6h. Western Blot analysis of HIF1α (115kDa) and B-ACTIN (45kDa) was done using 25µg protein lysate. The experiment was carried out three times independently revealing high variability of HIF1α levels and, thus, $P > 0.05$ for all comparisons. The average standardized optical density (SOD) of HIF1α (mean ± SD) is shown.

A


20% O2
10% O2
mM dbcAMP
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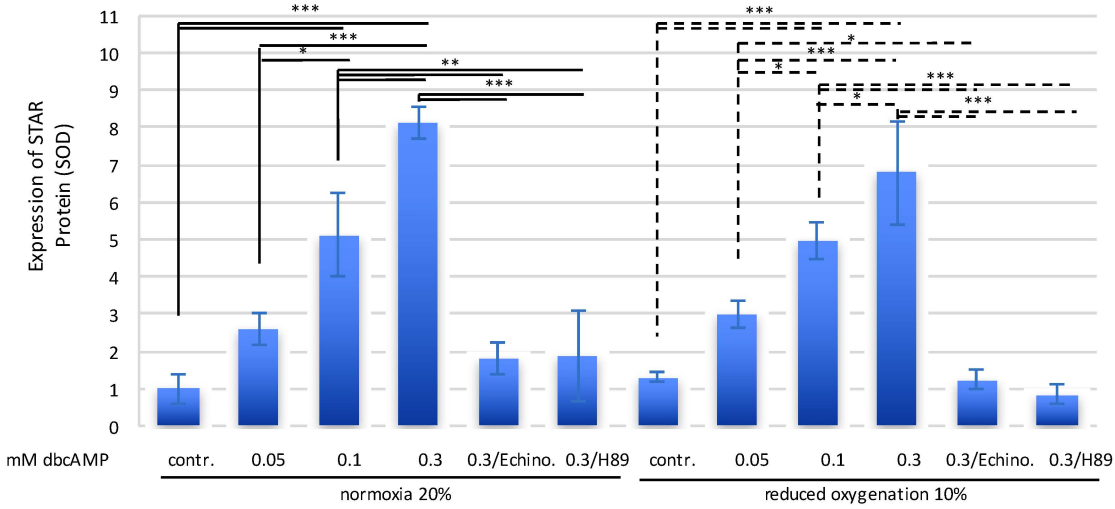
STAR
β-ACTIN


Expression of STAR Protein (SOD)
mM dbcAMP
contr.
0.05
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0.3/H89
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0.1
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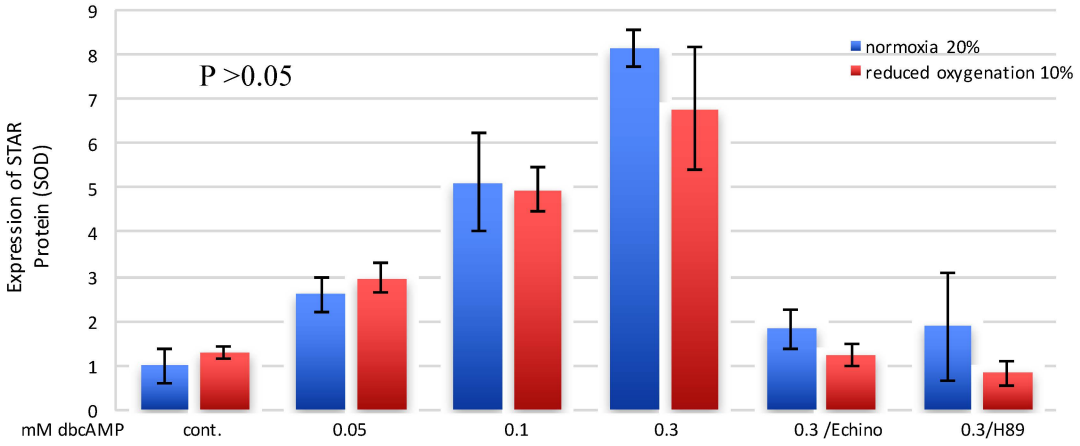
0.3/Echino.
0.3/H89
normoxia 20%
reduced oxygenation 10%


P >0.05
normoxia 20%
reduced oxygenation 10%
Expression of STAR Protein (SOD)
mM dbcAMP
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0.3
0.3 /Echino
0.3/H89

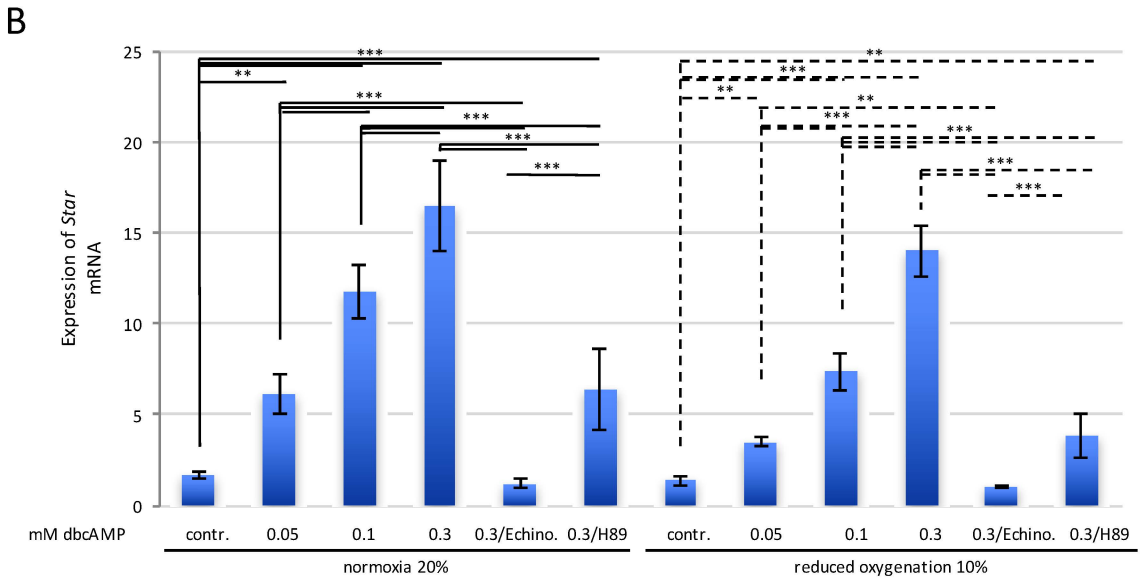

Figure 17: Effects of reduced oxygenation and HIF1α on the expression of STAR in mLTC1 Leydig cells. Cells were cultured for 6h in serum-free RMPI-1649 medium in presence of increasing doses of dbcAMP (0.05-0.3mM) under 20% and 10% O_2. The role of PKA was investigated by applying H89 (25µM), whereas the impact of HIF1 by treatment with echinomycin (5nM). All experiments were carried out three times independently. (A) A representative immunoblot of STAR protein is shown. B-ACTIN served as loading control. The Western Blot analysis was done by using 25µg of protein lysate. The average standardized optical density (SOD) for STAR (mean ± SD) is presented. (B) *Star* mRNA expression by real time (TaqMan) PCR. Different asterisk indicates (*) P <0.05, (**) P <0.01, (***) P <0.001.

8 Discussion

The gonads are physiologically exposed to reduced oxygen tension and hypoxia-inducible factor 1 α (HIF1α) is involved in the regulation of their normal activity such as the production of gametes and the provision of steroids (Alam, Maizels et al. 2004, Kim, Bagchi et al. 2009). Acting at the level of steroid acute regulatory protein (STAR), HIF1α was identified as a positive regulator of ovarian steroidogenesis in granulosa cells under reduced oxygenated conditions at 10% O_2 (Fadhillah, Yoshioka et al. 2014, Kowalewski, Gram et al. 2015). Nevertheless, little is known about the underlying molecular mechanisms. My master thesis examined downstream mechanisms focusing on the CREB and cJUN mediated effects under reduced oxygenated conditions (10% O_2) in KK1 granulosa cells. These investigations revealed that the HIF1 heterodimer transcriptional activity upregulates the expression and function of cJUN. It is also possibly involved in regulating the recruitment and binding capacity of P-CREB to the proximal *Star* promoter, leading to enhanced STAR expression under reduced oxygenated conditions (10% O_2). As a possible trans-mediator, the co-activator CBP/p300 was proposed to be involved in the HIF1-dependent recruitment of P-CREB. However, little information is available related to the impact of HIF1 heterodimer on the function of CBP/p300 in steroidogenic cells. Thus, affecting the function of P-CREB. The present thesis was designed to examine potential involvement of CBP/p300 by investigating its expression in steroidogenic cells under different oxygenated conditions. As in previous studies, also here KK1 granulosa cells were used as a steroidogenic model. They respond with high STAR expression to dbcAMP stimulation and generate high amounts of progesterone in a dosage-dependent manner. Interestingly, as shown previously, they also express clearly detectable levels of HIF1α under normoxic condition (Kowalewski, Gram et al. 2015) allowing for investigating its involvement in STAR-mediated steroidogenesis.

Expression of CBP

Because the detection of CBP/p300 in steroidogenic cells was associated with difficulties caused by high background staining generated from most commercially available antibodies, in this study, the appropriate Western Blot assay for validation of CBP/p300 expression has been established with the help of a CBP-HA-Tag expressing vector (pRc/RSV-m CBP-HA) (Chrivia, Kwok et al. 1993). The usage of an HA-Tag was found to be suitable to check transfection efficiency and was further used to identify both, the exogenously and endogenously expressed CBP. HA-Tag is a well-known epitope tag which does not interfere with the biological activity and function of recombinant tagged protein. It is a small peptide that has a size of only 1.1kDa and for this reason does not disturb the visualisation of the recombinant protein in Western Blot analysis.

The evaluation of CBP expression in KK1 cells revealed a decreased expression of the protein in echinomycin-treated cells. This finding was, indeed, interesting as for the first time it suggests a possible involvement of HIF1 heterodimer in regulating CBP availability in KK1 granulosa cells used as a steroidogenic model, by regulating its expression. Similar experiments were performed under normoxia (20% O_2) and reduced oxygenated conditions (10% O_2), yielding similar results.

CBP is recognized as key molecule involved in the communication between transcription factors and the basal transcriptional machinery (Vo and Goodman 2001), thus, playing an essential function in the integration of multiple incoming signals and in this way contributing to the appropriate transcriptional response (Kamei, Xu et al. 1996). Its crucial role is highlighted in the human Rubstein-Taybi Syndrom (RTS), in which a generalized dysregulation of gene expression caused by the loss of a single CBP allele results in deleterious multiple congenital malformations and mental retardation (Petrij, Giles et al. 1995). RTS is therefore the genetic evidence for the fact that the amounts of CBP within the cell are limited and shows the outcome of a relative small decrease of the cofactor. *In vitro* studies further confirmed the idea that the levels of CBP are restricted and lead to a competition between different transcription factors binding CBP (Hottiger, Felzien et al. 1998). The synergistic effects between transcription factors binding to CBP occur predominantly when the transcription factors share the same promoter as target, which corresponds to the situation at the proximal *Star* promoter (Kamei, Xu et al. 1996). Therefore, based on the herein presented results, it appears plausible that in steroidogenic cells HIF1 heterodimer directly affects the CBP availability as own binding partner and for other factors including P-CREB influencing, thereby, *Star* transcription under normoxia (20% O_2) and reduced oxygenated conditions (10% O_2). This assumption is supported by the fact that the transcriptional activity of P-CREB depends on the co-activating properties of CBP in order to initiate *Star* promoter activity (Manna and Stocco 2007). A model suggesting how the modulation of the co-activator availability can affect the response to extracellular stimuli, without the need of increasing transcriptions factors level or the stimuli input, was observed in primary mouse embryonic fibroblasts (MEF's) that have a conditional knockout of CBP/p300 (Kasper, Lerach et al. 2010). In that study, promoters rich in CRE binding sites were more resistant to the loss of the coactivator. However, to date, knowledge about the direct regulation of CBP expression is still restricted. Therefore, the herein proposed hypothesis regarding the direct regulation of CBP expression by HIF1 heterodimer and the involvement of this mechanisms in STAR-dependent steroidogenesis, warrants further elucidation.

Immunoprecipitation of CBP

In order to provide a basis for further investigations and address the hypothesis of the HIF1- and CBP-dependent recruitment of P-CREB to the proximal *Star* promoter, immunoprecipitation (IP) experiments were initiated in order the pulldown CBP using previously validated antibody. HEK cells were used as an appropriate model to establish the IP protocol, as they show high transfection efficiency regardless of vector size.

In these experiments, HA-tagged magnetic beads turned out to be the most efficient tool to isolate recombinant CBP after transient transfection of HEK cells with CBP-HA expressing vector. The magnetic separation is fast and avoids centrifugation steps. Thus, decreases the risk of destroying weak antibody-antigen binding and to lose the target protein. By using tagged magnetic beads, the specificity of the immunoprecipitation is secured and facilitates the process of the purification and isolation of CBP. At the same time an additional control vector, Flag-cJUNWT-Myc, was used to purify recombinant cJUN. In this part of the experiment, Flag-tagged agarose beads were applied. The IP protocol established in HEK cells will be tested in the future in transfected KK1 granulosa cells.

The functional implication of the established IP protocol gives rise to examine the functional interaction between CBP, HIF1α and P-CREB in KK1 granulosa cells in co-immunoprecipitation (co-IP) experiments both, under normoxia and reduced oxygenated conditions. It could be also applied in chromatin immunoprecipitation (ChIP) assay aiming to assess the association of protein complexes with the proximal *Star* promoter. This opens up the possibility to gain a better understanding of the regulatory mechanisms involved in CREB-mediated HIF1-dependent expression of STAR, giving possible answers to the postulated hypothesis of the HIF1-dependent recruitment of P-CREB to the proximal *Star* promoter, using CBP as a physical bridge. This proposed model is encouraged by the circumstance that both CREB and HIF1 complex bind to CBP (Arany, Huang et al. 1996, Freedman, Sun et al. 2002, Manna and Stocco 2007). The illustration of such complex and interaction of CBP with P-CREB and HIF1 was shown, e.g., on the *Ldha* (*lactate dehydrogenase A*) promoter and was suggested to be essential for the hypoxic response on the *Vegf* promoter (Firth, Ebert et al. 1995, Wu, Zhau et al. 2007). Furthermore, also the hypoxic induction of the murine neuroblastic glucose transporter (GLUT3) dependents on the interaction of CREB, HIF1α and CBP (Thamotharan, Raychaudhuri et al. 2013). In the latter study co-IP approach was used to purify the co-activator complex and was further verified by chromatin ChIP-assay, supporting our planned approach.

Effects of overexpressing cJUN in KK1 granulosa cells under reduced oxygenated conditions (10% O_2)

The expression and function of cJUN was identified to be directly regulated by the influence of HIF1 heterodimer as the inhibition of its function resulted in diminished levels of cJUN in KK1 cells (Lanfranchi 2016). In the present thesis, the expression of recombinant cJUN with a Flag-cJUNWT-Myc expression vector eliminated the effects induced by blockage of HIF1 activity through echinomycin, demonstrating a recovery of the expression and phosphorylation of cJUN on protein level. This encouraging result supports the role of HIF1-complex in regulating the expression and phosphorylation of cJUN. Our findings corroborate previously published observations in human carcinoma cell line (SiHa cells), showing that the hypoxic impact leads to increased levels of cJUN (Ausserer, Bourrat-Floeck et al. 1994), demonstrating thereby the requirement for the functional cooperation between cJUN and HIF1 heterodimer in the transcriptional regulation of hypoxic genes (Alfranca, Gutiérrez et al. 2002). Our observations are further supported by the reported dosage-dependent increase of *cJun* promoter activity in the TSGH8301 cancer cell line, in response to the overexpression of HIF1α (Chang, Liu et al. 2014). Nonetheless, the obtained results are still preliminary (low n-numbers of experiments) and investigations are still ongoing. Importantly, to finally prove the role of HIF1α in regulating cJUN-mediated expression of STAR, further experiments are planned that will include the assessment of the expression of STAR and steroidogenic output (P4) in cJUN-transfected cells.

Influence of reduced oxygenated conditions on STAR expression in mLTC1 Leydig cells

Additional experiments were carried out to examine the role of reduced oxygenated conditions (10% O_2) and HIF1α on the function and expression of STAR in Leydig cells. Despite the facts, that the testis is known to function physiologically under reduced O_2 tension and that several studies confirmed the positive impact of HIF1α on testosterone synthesis (Perkins, Hall et al. 1988, Lysiak, Kirby et al. 2009), the significance of HIF1α in STAR mediated testicular steroidogenesis was not yet clarified. In the present study immortalized murine mLTC1 Leydig cells were utilized as an appropriate experimental model. MLTC1 Leydig cell line was created from a clone of the Leydig cell tumour M548P which originated from a mouse of the C57B1/8 strain (Ascoli 1981, Rebois 1982). The cell line retained several functional characteristics of normal Leydig cells such as LH/hCG responsiveness, functional adenylate cyclase system and steroidogenic output (Rebois 1982). The steroidogenesis in mLTC1 Leydig cells is restricted to the production of progesterone (Rebois 1982). A possible disadvantage of using a cell line isolated from a tumour is that the cells are polyploid, having an increased number of chromosomes, and thus changes in steroidogenic response and genetic properties can occur with continual passage of the cells (Rebois 1982). Therefore, the capability of mLTC1 Leydig cells to produce steroids in response to dbcAMP was proved in control experiments by measuring the expression of STAR protein. MLTC1 Leydig cells showed a dose-dependent increase of STAR protein and the PKA dependence was validated by the application of H89, a selective blocker of the cAMP-PKA pathway which abolished the stimulatory effect of dbcAMP.

Based on the observations made in my master thesis in KK1 granulosa cells (Lanfranchi 2016), a comparative approach was chosen to examine the expression of STAR in mLTC1 Leydig cells incubated under 20% and 10% O_2. Treatment with echinomycin was used to evaluate the impact of HIF1 heterodimer. As expected, STAR protein expression increased in dosage dependent manner in response the dbcAMP treatment, but, unlike in KK1 cells, the reduced oxygenated condition did not affect the expression of STAR. However, similar to KK1 granulosa cells, the expression of STAR in mLTC1 Leydig cells appeared to be still under the control of HIF1, because a strong suppression of STAR was observed in presence of echinomycin under both conditions (20% and 10% O_2). This interesting result let us to speculate that mLTC1 Leydig cells express already high levels of HIF1α under normoxic conditions. Therefore, the expression of HIF1α protein was assessed under 20% and 10% O_2 confirming high constitutive presence of HIF1α in mLTC1 cells, which was not further strongly affected by the reduction of the oxygen concentration. This observation is consistent with examinations made *in vivo,* confirming the constitutive presence of HIF1α in murine Leydig cells (Lysiak, Kirby et al. 2009). However, at the same time, in the study by Lysiak et al. (2009), TM3 Leydig cells showed a dramatical increase of HIF1α in cells when culturing them under reduced oxygenation compared with normal (normoxic) culture condition. This discrepancy to our results can be due to the fact that the cell culture in the previous study (Lysiak, Kirby et al. 2009) was exposed to 5% O_2 for 24h, which represented a stronger hypoxic stress. Additionally, the TM3 Leydig cell line may have different sensitivity towards lower oxygen concentration. TM3 Leydig cells originate from non-tumorigenic primary epithelial cell culture, which underwent spontaneous immortalization *in vitro,* and respond to LH with an increase of cAMP

and cholesterol metabolism (Mather 1980). Thus, having a different genetic background, TM3 Leydig cells retained other aspects of *in vivo* Leydig cells than mLTC1 cells, which indeed may affect their responsiveness to reduced oxygenated conditions. Similarly, e.g. in breast cancer lines, the expression levels of HIF1α were linked to the proliferative and/or invasive potential of the respective cell line (Shi, Chang et al. 2010). Therefore, additional experiments applying exposure of mLTC1 cells to different O_2 levels are needed to assess their responsiveness to reduced O_2 concentrations and to determine the threshold of duration and O_2 concentrations needed to induce HIF1α expression with respect to STAR expression and steroidogenic performance. Future investigations are planned including another Leydig cell lines, e.g., MA-10 cells (Ascoli and Puett 1978).

Furthermore, another experimental variable has to be considered regarding both granulosa and Leydig cell cultures in investigations addressing hypoxia- and HIF1-mediated steroidogenesis. Thus, it has been proved that also the cell density influences the induction of reduced oxygenated condition in cell cultures (Wenger, Kurtcuoglu et al. 2015). It appears thus plausible that confluent steroidogenic cells may have higher oxygen consumption rates leading to higher stabilization of HIF1α.

Summary of the study

Taken together, the present work provides basis for better understanding of the role of CBP in the HIF1-mediated STAR expression in KK1 granulosa cells both, under normoxia (20% O_2) and reduced oxygenated conditions (10% O_2). The results obtained herein show a reliable method to demonstrate effectively the expression of CBP and to purify the protein for the analysis of possible protein interactions. Ultimately, these results and presented pilot experiments provide a useful tool for further investigations towards elucidating the contribution of HIF1 heterodimer to the regulation of the recruitment of P-CREB to the proximal *Star* promoter.

The most interesting discovery made from the study with functional implication is the downregulation of CBP expression during blockage of HIF1 heterodimer activity by the application of echinomycin. This implies a possible involvement of HIF1 heterodimer in the regulation of CBP availability within steroidogenic cells, representing an additional regulating loop affecting the entire gene transcription involved in steroidogenesis (Figure 18). Cumulatively, the presented study highlights the complexity of the functional interplay among different transcription factors involved in the in the initiation of *Star* transcription.

In addition, for the first time the role of HIF1α in STAR-mediated steroidogenesis was investigated in Leydig cells using mLTC1 model. Although the reduced oxygenation (10% O_2) did not affect the response of STAR to dbcAMP, similar to granulosa cells, STAR expression remained under the control of HIF1α. This implies a different sensitivity towards O_2 content in the culture condition.

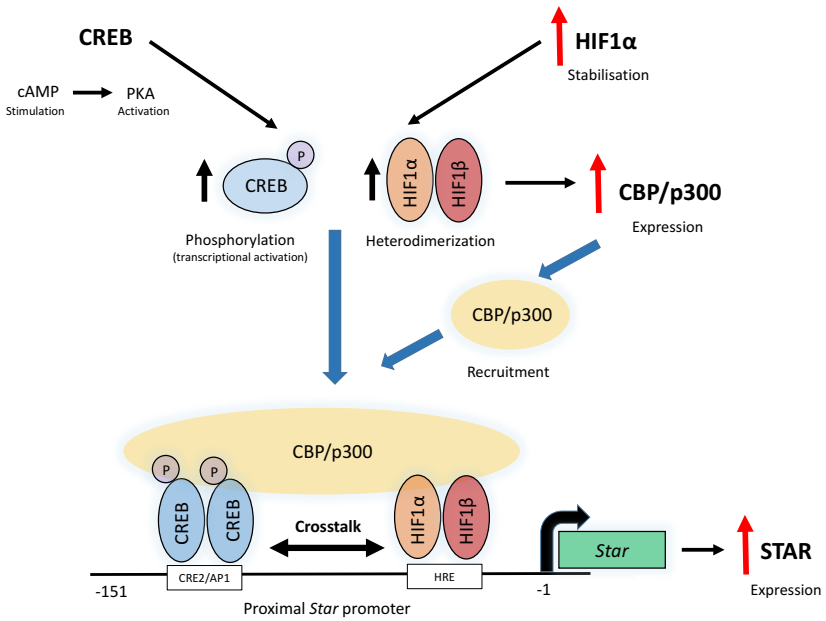

Figure 18: Proposed model of the HIF1-dependent regulation of STAR expression in KK1 granulosa cells (appears to apply to both normoxia (20% O_2) and reduced oxygenation conditions (10% O_2).

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10 Annex

10.1 Buffers and solutions

Annealing Buffer (sterile)

Tris 1M, pH7.5	400µl
EDTA 0.5M	80µl
NaCl 5M	400µl
Sterile Aqua bidest.	39.12ml

Formaldehyde 4%(Formalin 10%), buffered, pH 7.0 (according to Lillie)

40% formaldehyde	500ml
$NaH_2PO_4xH_2O$	20g
Na_2HPO_4	32.5g
Sterile Aqua bidest.	ad 500ml

NET-2 Lysis Buffer pH 7.4 (+4°C)

NaCl (300mM)	4.384g
Tris-HCl (50mM)	1.97g
Nonidet P (NP) - 40 (0.05%)	125µl
Sterile Aqua bidest.	ad 250ml

4x Sample Buffer for Western Blot (-20°C)

Sterile Aqua bidest.	16ml
0.5 M Tris pH 6.8	4ml
Glycerol	3.2ml
SDS 10%	6.4ml
β-mercaptothanol	1.6ml
0.2% bromphenol blue	32ml

Lower Tris Buffer pH 8.8

Tris Base	31.46g
Tris HCl	3.72g
SDS 10%	8ml
Sterile Aqua bidest.	ad 200ml

Upper Tris Buffer pH 6.8

Tris Base	0.182g
Tris HCl	3.72g
SDS 10%	2ml
Sterile Aqua bidest.	ad 50ml

Running Buffer 10x (used as 1x)

Tris Base	30.25g
Glycine	144.1g
SDS 10%	10g
Sterile Aqua bidest.	ad 1000ml

Transfer Buffer 10x

Tris Base	24.2g
Glycine	112.4g
SDS 10%	1g
Sterile Aqua bidest.	ad 1000ml

Transfer Buffer 1x

Methanol 10%	100ml
Transfer Buffer 10x	100ml
Sterile Aqua bidest.	ad 1000ml

TBS pH 7.6 10x

Tris	24g
NaCl	80g
Sterile Aqua bidest.	ad 1000ml

TBS-T (0.1% Tween 20)

TBS 10x	100ml
Sterile Aqua bidest.	ad 1000ml
Tween 20	1ml

PBS (phosphate buffered saline, 10x)

NaCl	80g
KCl	2g
Na_2HPO_4	14.4g
KH_2PO_4	2.4g
Sterile Aqua bidest.	ad 1000ml

PBS-T (0.25% Tween 20)

PBS 10x	100ml
Sterile Aqua bidest.	ad 1000ml
Tween 20	2.5ml

0.1 M Glycine pH 2.6

Glycine	1.5g
Sterile Aqua bidest.	ad 200ml

C100 Buffer

Hepes (20mM) pH 7.6	0.953g
Glycerol (20%)	40ml
KCl (100mM)	1.49g
MgCl (21.5mM)	0.41g
EDTA (0.02mM)	1.12mg
NP40 (0.02%)	0.04ml
Sterile Aqua bidest.	ad 200ml

MNase Buffer

0.3 M Sucarose	10ml (1.5M)
0.05M Tris pH 7.5	2.5ml (1.0M)
30mM KCl	500µl (3M)
7.5mMNaCl	75µl (5M)
4 mM $MgCl_2$	400µl (0.5M)
1mM $CaCl_2$	20µl (2.5M)
0.125% NP40	625µl (10%)
0.25% Na-deoxycholate	25µl/1ml of buffer
(Add Na-deoxycholate before usage)	

10.2 Chemicals and reagents

- 0.5% Trypsin-EDTA (10x) (Gibco, Thermo Fisher Scientific AG, Reinach, Basel, CH)
- 100 bp DNA Ladder (MBI Fermentas, Le Mont-sur-Lausanne, CH)
- 1kb DNA Ladder (MBI Fermentas, Le Mont-sur-Lausanne, CH)
- 2x Rapid Ligation Buffer, T4 DNA Ligase und T4 DNA Ligase, 3 U/µl (Promega, Dübendorf, CH)
- 40% Acrylamide/Bis Solution (MBI Fermentas, Le Mont-sur-Lausanne, CH)
- Ampicillin 100 µg/ml (Sigma Aldrich Chemie GmbH, Buchs, CH)
- Anti-FLAG M2 agarose beads (Sigma Aldrich Chemie GmbH, Buchs, CH)
- APS Ammonium Persulfate (BioRad Europe GmbH, Basel, CH)
- Bradford Quick Start (BioRad Europe GmbH, Basel, CH)
- Chloroform (Roth GmbH & Co., Arlesheim, CH)
- Chromatin Immunoprecipitation (ChIP) Assay Kit (Merck Millipore, Merck KGaA, Darmstadt, D)
- dbcAMP (2`0-dibutyryladenosin 3`5`cyclic monophosphate sodium salt) (Sigma-Aldrich Chemie GmbH, Buchs, CH)
- DifcoTM LB AGAR, Miller (Luria-Bertani) (Becton, Dickinson and Company, Spars, USA)
- DifcoTM LB BROTH, Miller (Luria-Bertani) (Becton, Dickinson and Company, Spars, USA)
- DMEM/Ham's F12 (Bioconcept Ltd, Allschwil, CH)
- DNA Loading Dye 6% (Fermentas GmbH, St. Leon-Rot, DE)
- Dulbecco's phosphate buffer saline (PBS), sterile (Eurobio, Les Ulis, Fr)
- Dynabeads^{TM} protein G kit (Thermo Fisher Scientific AG, Reinach, Basel, CH)
- E.coli XL1 Blue competent bacteria (Stratagene Europe, Amsterdam, NL)
- Ethanol (Roth GmbH & Co., Arlesheim, CH)
- Ethidiumbromid 1% (Roth GmbH &Co, Arlesheim, CH)
- FastStart Universal Probe Master Mastermix (Roche, Basel, CH)
- FBS (Fetal Bovine Serum) (Gibco, Thermo Fisher Scientific AG, Reinach, Basel, CH)
- Formaldehyde (Merck KGaA, Darmstadt, DE)
- FuGENE® HD Transfection Reagent (Roche, Basel, CH)
- GeneAmp® Gold RNA PCR Reagent Kit (Applied Biosystems, Rotkreuz, CH)
- Gentamicin sulfate (PAN Biotech GmBH, Aidenbach, DE)
- Glycerol Rotipuran®: 99.5% water-free (Roth GmbH & Co., Arlesheim, CH)
- Glycine (Sigma Aldrich Chemie GmbH, Buchs, CH)
- Goat Anti-Rabbit (Vector Laboratories, Burlingame, USA)
- HCl (Merck KGaA, Darmstadt, DE)
- Hind III und Not I, NE Buffer 3.1(New England Biolabs Inc, Frankfurt a.M., DE)
- Isopropanol (2-Propanol, Sigma Aldrich Chemie GmbH, Buchs, CH)
- KCl (Merck KGaA, Darmstadt, DE) ·
- KH_2PO_4 (Merck KGaA, Darmstadt, DE)

- L-Glutamine 200mM (100x) (Gibco, Thermo Fisher Scientific AG, Reinach, Basel, CH)
- Methanol (Merck KGaA, Darmstadt, DE)
- $Na_2HPO_4xH_2O$ (Merck KGaA, Darmstadt, DE)
- NaCl (Merck KGaA, Darmstadt, DE)
- Nonidet P (NP)-40 (Sigma Aldrich Chemie GmbH, Buchs, CH)
- Penicillin/Streptomycin (100x) (Sigma Aldrich Chemie GmbH, Buchs, CH)
- Pierce Anti-HA magnetic beads (Thermo Fisher Scientific AG, Reinach, Basel, CH)
- PrecisionPlusProteinTM, Dual Color Standards (BioRad Europe GmbH, Basel, CH)
- Proteinaseinhibitor (10µl/ml Buffer, Sigma Aldrich Chemie GmbH, Buchs, CH)
- Qiagen Plasmid Maxi Kit and Qiaex II Gel Extraction Kit (Qiagen AG, Hombrechtikon, CH)
- Reverse Transcriptase (Applied Biosystems, Rotkreuz, CH)
- Riboloc RNase Inhibitor, 40U/µl (Fermentas, Le-Mont-sur-Lausanne, CH)
- RPMI 1640 (Bioconcept Ltd, Allschwil, CH)
- RQ1 DNase RNase-Free (Promega, Dübendorf, CH).
- SDS (Sodiumdodecylsulfat) (Sigma Aldrich Chemie GmbH, Buchs, CH)
- Sodium hydrogencarbonate (Sigma Aldrich Chemie GmbH, Buchs, CH)
- SuperSignal®-West-Femnto/Pico Plus - Maximum-Sensitivity-Substrate (Thermo Fisher Scientific AG, Reinach, Basel, CH)
- TEMED (Tetraethylenmethylendiamin) (BioRad Europe GmbH, Basel, CH)
- Tris Base (Trizma® base, Minimum 99,9% Titration) (Sigma Aldrich Chemie GmbH, Buchs, CH)
- Tris-HCl (Trizma® Hydrochlorid, Reagent Grade) (Sigma Aldrich Chemie GmbH, Buchs, CH)
- TRIzol® (Ambion Life Technologie, Thermo Fisher Scientific AG, Reinach, Basel, CH)
- Tween 20 (Sigma Aldrich Chemie GmbH, Buchs, CH)
- UltraPure Agarose (Thermo Fisher Scientific AG, Reinach, Basel, CH)
- β-Mercaptoethanol (Sigma Aldrich Chemie GmbH, Buchs, CH)

10.3 Consumables supplies and equipment

- 12 tube magnet (Qiagen AG, Hombrechtikon, CH)
- 6-well plates Tissue culture test plates (TPP® Trasadingen, CH)
- 7500 Fast Realtime PCR System (Applied Biosystems, Rotkreuz, CH)
- 96-well optical plate (Applied Biosystems, Rotkreuz, CH)
- Cell Scarper 24mm (TPP®, Trasadingen, CH)
- Centrifuge tubes 15, 50ml (TPP®, Trasadingen, CH)
- CO_2 Incubator C16 (Labotect, Labor Technik Göttingen, Göttingen, Germany).
- Criterion BlotterTM, (BioRad Europe GmBH, Basel, CH)
- Immunoblot PVDF Membrane (BioRad Europe GmBH, Basel, CH)
- Mini Protean& Tetracell (BioRad Europe GmBH, Basel, CH)
- Molecular Imager ChemiDocTM XRS+ (BioRad GmBH, Basel, CH)
- Owl easy castTM B1A (Thermo Fisher Scientific AG, Reinach, CH)
- Petri dishes (Sterilin Limited, Newport, UK)
- Semimicrovolume cuvette, quartz, (BioRad Europe GmBH, Basel, CH)
- Software GraphPad3 (GraphPad Software Inc., San Diego, California, USA)
- Sonicators (Homogenisator VDI 12, VWR, Dietikon, CH)
- Thermocycler (Mastercycler, Vaudaux-Eppendorf AG, Basel, CH)
- Thermomixer Thermostat plus, (Vauxdaux-Eppendorf, Basel, CH)
- Tissue culture flask150 (TPP®, Trasadingen, CH)
- UV-Spectrophotometer (Smart SpecTM Plus, BioRad, Reinach, CH) ·
- Forma Steri cycle CO_2 Incubator (Thermo Fisher Scientific AG, Reinach, CH)

11 Acknowledgements

Hereby I would like to gratefully acknowledge everybody who, in one or another way, contributed in accomplishing this present doctoral thesis and without whom this thesis could not have been arisen and finally completed.

First of all, I would like to thank my supervisor and the Director *ad interim* of the institute, Prof. Dr. Mariusz Kowalewski, for his guidance, his scientific expert advice and his encouragement throughout this thesis process. Not to forget, I would like to thank him very much for his careful proof-reading and for giving me the chance to work in his laboratory and become part of his team.

I would like to express my great appreciation to my co-examiner, Prof. Dr. Max Gassmann, for his generous support in developing this present doctoral thesis. His willingness to give his time, knowledge, and help has been very much appreciated by me.

I would like also to thank the former head of the institute Prof. Dr. Alois Boos for his continuous support and acknowledgment of my work in his former institute, laboratory, and as a teaching assistant in the practical courses for veterinary students.

I am particularly grateful for the assistance given to me by Dr. Aykut Gram. He advised, helped, and supported me in every situation and gave me enthusiastic encouragement.

Here I would like to extend my sincere appreciation to Dr. Marta Nowak, PhD. Her expert advice and explanations, her valuable, never-ending loyalty, and empathy kept me on track during the whole process of completing the thesis.

The technical expertise, contribution, and great kindness of Ricardo Fernandez Rubia, the technician in the molecular and cell biology laboratory of the institute, was very much appreciated.

I would like to express more my sincere gratitude to all the people, colleagues and (former) co-workers of the Institute of Veterinary Anatomy for their help, kindness, enthusiasm, and understanding, especially to Elisabeth Högger, Doris Herzog, Miguel Tavares Pereira, Tina Gysin, Ashlihan Turhan, Katrin Süss, and Orsolya Balogh.

Furthermore, I would like to thank Prof. Dr. Raffaella Santoro of the Department of Molecular Mechanisms of Disease for her constructive expert advice and great generosity allowing me to complete some experiments in her laboratory. Herewith, I would like to extent my great appreciation to Marcin Damian Roganowicz, PhD student in the Department of Molecular Mechanisms of Disease, for his kind help, enthusiasm, and guidance throughout completing parts of my research work.

Finally, I wish to express a deep thank you to my family, friends, and colleagues for their highly valuable encouragement, understanding, and endless cheering up, supporting me throughout the research work and the final steps of completing this present doctoral thesis.

Many Thanks to all of you!

12 Curriculum Vitae

Vorname Name	Bettina Lanfranchi
Geburtsdatum	04.12.1992
Geburtsort	Samedan, CH
Nationalität	Schweiz
Heimatort bei Schweizer/-in	Poschiavo, CH
08/1999 – 07/2005	**Grundschule,** Primarschule, Silvaplana, CH
08/2005 – 07/2011	**Gymnasium,** Lyceum Alpinum Zuoz, Zuoz, CH
29.06. 2011	**Matura**, Leistungsfächer: Mathematik/Physik, Chemie Lyceum Alpinum Zuoz, Zuoz, CH
09/2011 – 09/2016	**Studium Veterinärmedizin,** Schwerpunktfach: Biomedizinische Forschung Vetsuisse Fakultät Zürich, Zürich, CH
17.01.2017	**Eidgenössische Prüfung Veterinärmedizin,** Vetsuisse Fakultät Zürich, Zürich, CH
04/2017 – 08/2019	**Anfertigung der Dissertation** unter Leitung von Prof. Dr. Mariusz P. Kowalewski, PhD am Veterinär-Anatomischen Institut der Vetsuisse-Fakultät Universität Zürich Direktor *ad interim*: Prof. Dr. Mariusz P. Kowalewski, PhD